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Analysis of genomic and transcriptomic studies of Alzheimer’s Disease to identify novel pathways for study in Drosophila melanogaster

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Analysis of genomic and transcriptomic studies of Alzheimer's Disease to identify novel pathways for study in *Drosophila melanogaster*

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Declaration

I hereby declare that this thesis is the results of my own investigations, except where otherwise stated. All other sources are acknowledged by bibliographic references. This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree unless, as agreed by the University, for approved dual awards.

I confirm that I am submitting this work with the agreement of my Supervisor(s).

Yr wyf drwy hyn yn datgan mai canlyniad fy ymchwil fy hun yw'r thesis hwn, ac eithrio lle nodir yn wahanol. Caiff ffynonellau eraill eu cydnabod gan droednodiadau yn rhoi cyfeiriadau eglur. Nid yw sylwedd y gwaith hwn wedi cael ei dderbyn o'r blaen ar gyfer unrhyw radd, ac nid yw'n cael ei gyflwyno ar yr un pryd mewn ymgeisiaeth am unrhyw radd oni bai ei fod, fel y cytunwyd gan y Brifysgol, am gymwysterau deuol cymeradwy.

Rwy'n cadarnhau fy mod yn cyflwyno'r gwaith hwn gyda chytundeb fy Ngoruchwyliwr (Goruchwylwyr)

Signature: A.W. Jones

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Abstract

Alzheimer's disease (AD) is a neurodegenerative disease with a mostly unknown aetiology, that is characterised by accumulation of the abnormal proteins, amyloid-beta and microtubule associated protein tau (tau). Tau is particularly interesting due its involvement in a range of neurodegenerative disorders collectively known as tauopathies. In recent years, our understanding of the mechanisms of tauopathies has greatly expanded due to two factors: 1) the use of model organisms like *Drosophila melanogaster*, *C. elegans* and mouse have provided powerful experimental platforms to analyse the cellular and molecular events of disease, and 2) genomic and transcriptomic methodologies have identified novel targets and molecular mechanisms that can then be further tested in model organisms. This thesis builds on these developments by undertaking an analysis of published transcriptomic and genomic studies to identify novel conserved pathways and proteins that play a key role in human disease and have clear orthologues in *Drosophila*. In this way my study seeks to identify novel approaches to using *Drosophila* to provide new insights into the mechanism of neurodegenerative disease.

To do this, I analysed 52 published studies using genomic and/or transcriptomic methods to reveal the genetic changes that underlie AD. From this survey, I identified the most significant genes identified in each study for further detailed analysis and determined their human and *Drosophila* orthologues. The most frequently occurring genes were scored, ranked and placed into protein-protein interaction networks (PPIN) using Cytoscape. Using Gene Ontology (GO) enrichment analyses, functional annotations of both species' whole networks were acquired and examined. Centrality analysis was used to identify potential candidate proteins conserved in both humans and *Drosophila*. Using the Cytoscape plugin GASOLINE, conserved protein modules were identified and analysed. My results found

significant enrichment of functions and genes/proteins primarily relating to vesicle processing, protein kinase processes, and RNA splicing. Individual proteins with high-degree, high-betweenness values in both species, such as SRC/Src64B and EGFR/Egfr, and protein complexes enriched in biological processes such as exocytosis, membrane dynamics, and RNA splicing are exciting potential candidates for further studies concerning tau-based pathology. Together, my work complements previous findings in the field while providing novel insights into the connections between disease-causing genes and mechanisms in tauopathies such as AD.

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Introduction

Dementia & Alzheimer's Disease

Dementia is an umbrella term referring to a group of neurological disorders primarily characterised by the progressive impairment and eventual loss of cognitive functions and neuronal deterioration (Ritchie & Lovestone, 2002). The global prevalence of dementia is estimated to be approximately 46.8 million people (Prince et al., 2015), and in Britain alone, this number is predicted to rise to 1,205,000 by 2040 (Ahmadi-Abhari et al., 2017). Dementia also bears a significant economic burden with costs estimated to increase from $818 billion (USD) to $1 trillion by 2018 (Wimo et al. 2016). As the world's population ages, both the incidence and economic impact of dementia will inevitably increase.

Of the dementias, Alzheimer's disease (AD) is the most commonly diagnosed form. AD is a chronic neurodegenerative disease, and most cases are sporadic with a less well understood aetiology. Familial forms of Alzheimer's Disease (FAD) are caused by mutation in one of three genes: *PSEN1*, located on chromosome 14; *PSEN2*, located on chromosome 1; and *APP*, located on the long arm of chromosome 21. Both sporadic and familial forms converge upon similar clinical systems, which are memory dysfunction and loss, beginning with declarative memory impairment in the early stages of the disease before eventually resulting in widespread amnesia and inability to store new information. As the disease progresses, impairments in language, judgement, attention and other executive functions become apparent. Psychological and behavioural sequelae also become more prominent and severe, and may include depression, delusions, hallucinations, and increased aggression. In late-stage AD, almost all cognitive functions have been lost, and individuals are completely dependent on caregivers. At this stage death occurs, though usually as a result of an infection or other physical morbidity rather than AD itself.

AD is a neurodegenerative disease, and its neuropathology is marked by profound alterations in neuronal structure and function. It is characterized by the progressive deterioration of neuronal subsets within specific brain regions, most severely affecting regions involved in memory first, before spreading to connected cortical regions. This progressive deterioration is correlated with neuronal aggregation of hyperphosphorylated microtubule associated protein tau, a protein required for microtubule assembly and stability, into tangles (Wood et al., 1986; Alonso et al., 1996) and extracellular aggregation of amyloid-beta (Aβ) peptides into plaques (Sadigh-Eteghad et al., 2015). It is now the general consensus that the pathophysiology of AD is primarily caused by pathogenic variants of these two biomolecules; however, recent research suggests tau is more responsible for the cognitive and clinical symptoms than Aβ (Giannakopoulos et al., 2003; Cho et al., 2016).

Amyloid-β

Aβ is a peptide formed via sequential cleavage of the transmembrane protein amyloid precursor protein (APP) by the proteases β- and γ-secretase (Lichtenthaler et al., 2011). Briefly, β-secretase cleaves APP at the N-terminus, resulting in the formation of two derivatives: soluble APP (sAPPβ) and β-C-terminal fragments (CTFβ); the former is released into the extracellular space, while the latter remains tethered to the cell membrane. CTFβ is then cleaved by γ-secretase, resulting in Aβ (Thinakaran & Koo, 2008; Chen et al., 2017). However, this process is imprecise, and depending on the site γ-secretase binds to, different Aβ isoforms are produced. Of these isoforms, $A\beta_{40}$ and $A\beta_{42}$ are the most abundant (Portelius et al., 2010), the latter considered to be the primary toxic variant (Pike et al., 1993; Vadukul et al., 2017).

It is suggested that increased levels of Aβ are the primary cause of AD, and that deposition of this toxic Aβ into the extracellular space prompts a “chain reaction” leading to profound

pathological changes in AD; this is known as the amyloid cascade hypothesis (Hardy & Higgins, 1992). This idea was initially based on two observations: 1) Aβ forms the bulk of the extracellular depositions known as senile plaques (SP), a key histopathological feature of AD (Dickson, 1997); and 2) studies on familial AD (FAD) revealing that mutations in the *APP* and *PSEN* genes lead to increased levels of Aβ, particularly $A\beta_{42}$ (Duff et al., 1996; Kumar-Singh et al., 2006). In recent years the hypothesis has been amended to emphasise soluble Aβ oligomers as the primary drivers of AD. Formed via the oligomerisation of Aβ isoforms (El-Agnaf et al., 2000), there is evidence to suggest that these oligomers exhibit significant synaptotoxicity, particularly at excitatory synapses (Lacor, 2004; Koffie et al., 2009).

Tau Protein

Tau refers to a group of proteins encoded by the microtubule-associated tau protein gene (*MAPT*), located on chromosome 17q21 in humans (Wade-Martins, 2012). The primary physiological function of tau is to stabilise microtubules by binding to tubulin regions on the microtubule structure (Hirokawa et al., 1988; Makrides et al., 2004; Kadavath et al., 2015) via its MT-binding repeats (Cleveland et al., 1977; Gustke et al., 1994). Alternative mRNA splicing of exons 2, 3 and 10 in *MAPT* results in the production of six tau isoforms, the structures of which are dependent on the inclusion or exclusion of some or all of these exons during mRNA translation (Buée et al., 2000). Exons 2 and 3 are translated into N1 and N2 regions of the N-terminus, respectively, while exon 10 translates into the R2 aspect of the C-terminus microtubule-binding repeat domain (Liu & Gong, 2008). Figure 1 illustrates the different isoforms and their structures.

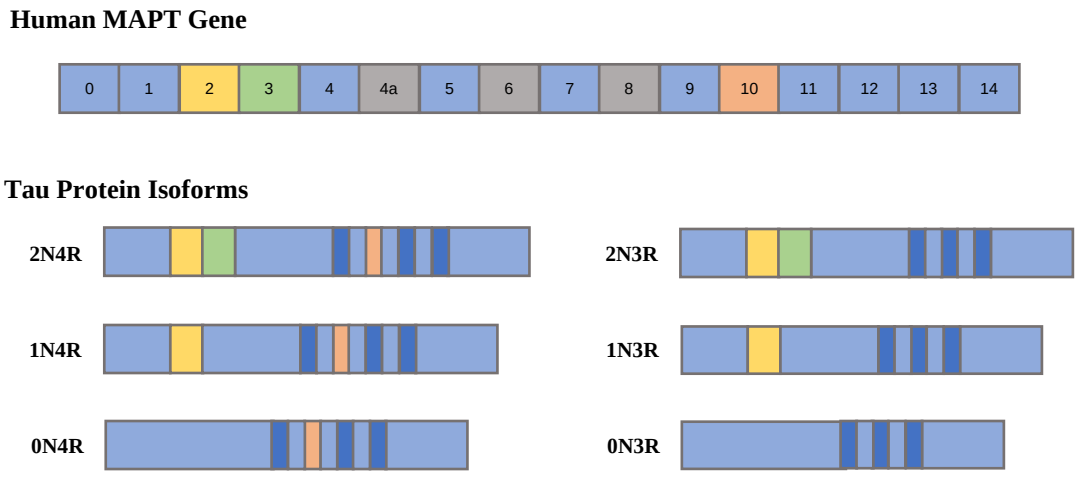


Figure 1. Human MAPT gene and the six tau isoforms. Alternative splicing of exons 2 (yellow), 3 (green) and 10 (orange) results in the formation of the six tau isoforms. Exons 4a, 6, and 8 (grey) are not transcribed in the human brain. Dark blue bars represent microtubule-binding repeats and the yellow and green bars represent the N-terminal domains. Adapted from Hefti et al., (2018). The six human tau isoforms differ in their primary structure in terms of the presence and absence of two N-terminal domains, and the three or four carboxy-terminal microtubule binding domains.

The isoforms are typically categorised by the number of MT-binding repeats, i.e., 3R or 4R. 4R tau binds more strongly than 3R as a result of its larger number of MT-binding repeats (Panda et al., 2003). Tau also promotes the assembly of microtubules by affecting tubulin polymerisation (Weingarten et al., 1975; Drechsel et al., 1992; Li & Rhoades, 2017), and has been implicated in axonal development due to an increased presence at the distal ends of developing neurons (DiTella et al., 1994; Kempf et al., 1996).

Tau function is regulated by post-translational modifications, and as a phosphoprotein it is particularly susceptible to phosphorylation. Phosphorylation of tau, typically by kinases and phosphatases such as GSK3β, affects its binding affinity for microtubules, allowing microtubules to remain dynamic and preventing inhibition of axonal transportation (Liu et al., 2007; Cuchillo-Ibanez et al., 2008; Rodríguez-Martín et al., 2013). Tau phosphorylation is

also elevated during embryonic development, possibly to promote axonogenesis and plasticity (Brion et al., 1994; Biernat & Mandelkow, 1999; Yu et al., 2009). Tau is modulated by other post-translational processes, such as glycation and nitration, but their significance is yet to be clarified (Ballatore et al., 2007).

It is generally believed that tau becomes pathogenic as a result of hyperphosphorylation, where phosphoryl binding sites on its structure become saturated. More specifically, normal tau contains 2-3 mol phosphate/mol protein, whereas hyperphosphorylated tau contains approximately 6-8 mol phosphate/mol protein (Ksiezak-Reding et al., 1992; Köpke et al., 1993). The cause of hyperphosphorylation is unclear, but amongst other proposals it has been suggested that aberrances in the activities of kinases and phosphatases, normally responsible for regulating tau phosphorylation, could be responsible (e.g. Nuydens et al., 1995; Sato et al., 2002). When hyperphosphorylated, tau assembles to form insoluble filaments, namely paired helical filaments (PHFs) and straight helical filaments (SHFs) (Crowther & Goedert, 2000; Alonso et al., 2001). These filaments are composed of two protofilaments, with cross-β/β-helix structures and cores consisting of the tau amino acids 306–378, i.e. R3, R4, and 10 residues following R3 (Berriman et al., 2003; Fitzpatrick et al., 2017; Falcon et al., 2018). Both PH and SH filaments aggregate to form the bulk of NFTs, dense intracellular structures that typically occur within the axonal processes of neurons (Bancher et al., 1989).

Tau Pathogenicity & *Drosophila*

As examining the effects of tau in humans *in vivo* is both practically and ethically difficult, most of our knowledge on tau, both normal and abnormal, derives from animal models of AD. A wide variety of animal organisms can be used to simulate AD, including *Mus musculus* (house mouse; Elder, Sosa, & Gasperi, 2010), *Danio rerio* (zebrafish; Newman, Ebrahimie, & Lardelli, 2014), and *Caenorhabditis elegans* (nematode; Alexander, Marfil, & Li, 2014). In recent years, *Drosophila melanogaster* (fruit fly) has come to the fore as an AD

model, especially pertaining to tau-specific pathology. A review by Sivanantharajah, Mudher, & Shepherd (2019) highlights the advantages of *Drosophila* for modelling AD and other tauopathies, including its simplicity, cost-effectiveness (compared to other model organisms, particularly vertebrates) and genetic malleability. More importantly, its genome is evolutionarily well-conserved, with 60% of genes estimated to be conserved between it and humans. Interestingly, 75% of known human disease genes have a homologue in *Drosophila*, including tau (Reiter et al., 2001).

This is important because, while abnormal tau's pathogenicity is well-documented, the specific mechanisms behind its pathogenicity and subsequent propagation is still largely unknown. As previously stated, it is generally believed that hyperphosphorylation is responsible for tau pathogenicity, and wild-type *Drosophila* tau too undergoes both normal and abnormal phosphorylation by various protein kinases (Chatterjee, Sang, Lawless, & Jackson, 2009; Yeh et al., 2010). However, the questions of why it becomes hyperphosphorylated and whether hyperphosphorylation is the sole inducer of tau pathology remain largely unanswered. Hypothetically speaking, given that hyperphosphorylation of native tau is present in both species, the pathways or processes that satisfy the criteria of these questions should also be evolutionary conserved to some degree. This is further emphasised by both the identification and characterisation of proteins and biological processes that have been found to exacerbate AD pathology, such as BIN1 and endocytosis (Cataldo et al., 1997; Cataldo et al., 2001), across various animal models, including *Drosophila*. Numerous genomic and transcriptomic studies have also demonstrated the expression patterns of dozens of proteins to be significantly altered in tauopathy models. However, a survey of the current literature indicates no prior across-species attempt to identify, examine, and underline the most significant proteins from these studies as both individual candidate proteins and indicators of wider biological pathways that may contribute

to AD pathology. In this study, I will attempt to achieve this objective by using the following methods:

1. I will conduct a literature search and identify genomic and transcriptomic studies that meet the designated criteria, as further detailed in the "Methods" section.
2. I will examine identified studies to identify the top ten (± n) most significant proteins/genes, determine their human and *Drosophila* orthologues, and compile them for further analysis.
3. I will create two protein-protein interaction networks for both species using the results from the previous step and conduct a Gene Ontology (GO) enrichment analysis to identify the most significant global functions amongst the proteins.
4. Using various tools in Cytoscape, I will highlight the most topologically interesting individual proteins in both species using criteria set by Yu et al (2007).
5. Using the Cytoscape plugin GASOLINE, I will search for and identify conserved protein modules/complexes that share similar biological functions.

From this work, I hope to identify BOTH novel candidate proteins and biological processes and highlight proteins and pathways already known to be associated in AD that may be further explored in future studies.

Methods

Literature Search

All work presented in this thesis is based on studies found in a search of the primary literature in academic publication databases. The databases used were Google Scholar and Bangor University Library. Literature searches were made using the primary search term *Alzheimer* in combination with relevant key words, such as *Drosophila*, *genomics*, *genes*, *microarray*, and *GWAS*, to filter and specify the most relevant studies. The search period was from January 2000 to August 2020. Primary research articles were prioritised and reviews were excluded, although publications describing meta-analyses of relevant data and research were included. In total, 52 papers were selected and analysed in this study. *Homo sapiens* was the most prominent organism across all studies, followed by, in order, *Drosophila melanogaster*, *Mus musculus*, and *Rattus norvegicus*. For each organism, cited genes were converted to human and/or *Drosophila* orthologs using UniProt (Bateman et al., 2020) and the HUGO Gene Nomenclature Committee (Braschi et al., 2018).

Identification of Most Significant Genes

Data from selected papers and supplementary content (where available) were analysed to identify genes that appeared to have significant changes in expression profiles in association with AD or other tauopathies. The most significant genes in each paper were selected according to a number of criteria. For most articles, the key criterion for selecting a gene were those that showed the most significant differences in expression in AD compared to controls, i.e., genes with low p-values (<0.05), in the published statistical analyses. These genes were selected, ranked by level of significance, and entered into an Excel spreadsheet. Other conditions used to select genes for the study included fold differences (FD), i.e., genes whose expression changes were in excess of ±1.50, and q-values (adjusted p-values taking

into account a false discovery rate (FDR)) equalling <0.05. It should be noted that these measures were primarily used if multiple genes either shared identical p-values or had no such values presented within the research. If a paper listed no quantitative measures of significance, then genes were selected based either on genes previously identified as significant or prominent and/or identified as important by the authors.

Protein-Protein Interaction Networks Generation & Analysis

To generate the protein-protein interaction networks (PPINs), the Search Tool for the Retrieval of Interacting Genes/Proteins database (STRING v11.0 (Szklarczyk et al., 2019) was employed. STRING is an online biological database containing information on known and theoretical protein-protein interactions. STRING imports protein interaction data from numerous sources, including experimental data, datamined literature, and computational predictions. This data is subsequently weighted and incorporated to calculate a confidence score for all protein interactions. For this project, genes identified from the literature searches were inputted as a list and two networks were constructed: one for *Homo sapiens*, and the other for *Drosophila melanogaster*.

Putative orthologs of each gene were identified using the HGNC Comparison of Orthology Predictions search (HCOP; Eyre, Wright, Lush & Bruford, 2006), a freely available tool integrating ortholog predictions from several databases for human genes. As some genes from the literature search were derived from different organisms, each one was converted to its approximate human equivalent using HPOC. These results were then imported into Excel and filtered to ensure that orthologs with the strongest evidence (i.e., those with multiple concurring databases) were kept. *Drosophila* orthologs (if available) were identified and filtered via Excel.

Both networks used the same settings: edges were based on confidence scores, all options for interaction sources were selected, and the minimum required interaction score was set to "medium confidence (0.400)". Once generated, these networks were imported into Cytoscape, a freely available bioinformatics application, using the stringApp plugin (Doncheva, Morris, Gorodkin & Jensen, 2018).

Gene Set Enrichment Analysis

To understand the overall relevance of identified genes to AD pathology, their functions must be described. To achieve this, gene set enrichment analyses (GSEA; Subramanian et al., 2005) were conducted. GSEA, also known as functional enrichment, attempts to determine the presence of significantly over-represented sets of genes in a gene list, in comparison to background set of genes (in this case, an organism's genome). These sets are typically, though not always, functionally related in a biological pathway.

Briefly, GSEA consists of three primary steps:

1. Calculation of an Enrichment Score (ES). The ES reflects the amount of overrepresentation in a gene set at the extremes (i.e., top and bottom) of the list.
2. Estimation of statistical significance of ES. This is calculated via a phenotype-based permutation test, where a null distribution for the ES is then generated.
3. Adjustment for multiple hypothesis testing. Enrichment scores for each set are normalised and the false discovery rate (FDR) is computed.

GSEA was undertaken for both networks using stringApp. Gene Ontology terms were retrieved as functional categories, namely molecular function, biological process, and cellular component. Enrichment results were then filtered to remove redundant terms (i.e., terms too similar to previous, higher-scoring terms) using a cut-off value of 0.5.

Network Centralities Analysis

Given a large network containing multiple nodes with numerous interactions, identifying the most important of these nodes in regard to network structure and information flow is vital. Measuring a node's centrality is one such method by which a node's importance in a network can be estimated. Following Freeman's (1978) three formalised centrality measures (degree, closeness, and betweenness), numerous centrality measures have been formulated in order to ascertain influential nodes.

Within the context of protein interaction networks, there is a well-established relationship between connectivity and essentiality, whereby proteins with higher levels of connectivity are more essential than those with lower levels, and their removal lethal for an organism (Jeong, Mason, Barabási & Oltvai, 2001; Hahn & Kern, 2004). Therefore, characterising the most central proteins in a disease network may reveal which proteins are more likely to be causative factors in a pathological process.

Analyses of centrality measures for each network were performed using the CentiScaPe plugin (Scardoni, Petterlini & Laudanna, 2009). A brief description of each measure (degree, betweenness, closeness, eccentricity, radiality, centroid value, stress) and their mathematical formulae follow.

Degree

The most basic topological index, the degree of a node, is determined by the number of direct connections to other nodes. Nodes with higher degrees are more central and are more likely to act as hubs for other nodes with lower degrees. In biological terms, degree can allow for an estimation of a protein's importance within a protein-protein interaction network.

Closeness

$$C_{clo}(v) := \frac{1}{\Sigma_{w \in V} dist(v, w)}$$

The closeness centrality ($C_{clo}(v)$) of a node is determined by calculating the shortest paths between the node and all other nodes, then assigning each node a score based on their average distance. Nodes with high closeness values are in close proximity to other nodes and are also more likely to be more central within a network. In contrast, nodes with low closeness values are more distant from other nodes, and less likely to be central within a network.

A protein with a high closeness value in a protein network can be interpreted as having functional relevance for other proteins, possibly regulating their activities. However, it may also be irrelevant for some proteins, with little to no influence on their functioning.

S.-P. Betweenness

$$C_{spb}(v) := \sum_{s \neq v \in V} \sum_{t \neq v \in V} \delta_{st}(v)$$

Where

$$\delta_{st}(v) := \frac{\sigma_{st}(v)}{\sigma_{st}}$$

The betweenness value of a node is calculated by considering couples of nodes (*v1*, *v2*) and counting the number of shortest paths linking *v1* and *v2* and passing through node *n*. Next the value is related to the total number of shortest paths linking v1 and v2. Then, the value is related to the total number of shortest paths linking *v1* and *v2*. As a result, a node with a high betweenness score is essential in maintaining node connections for certain paths; removal of such a node would disrupt communication within a network.

In a biological context, betweenness can indicate the role of a protein in facilitating communication between other proteins. In signalling pathways, for example, proteins with high betweenness values are likely to maintain signalling mechanisms between distant proteins.

Local Network Alignments

The purpose of local network alignment (LNA) is to identify potentially similar subgraphs of between networks. Within a protein-protein interaction network, these subgraphs can be interpreted as evolutionary conserved protein complexes. Here I used the Cytoscape plugin GASOLINE (reference), which computes and visualises multiple local alignments using the eponymous algorithm (Greedy And Stochastic algorithm for Optimal Local alignment of Interaction Network; reference).

The human and *Drosophila* orthologs obtained previously were used to retrieve protein sequences from UniProt and FlyBase, respectively. The sequences were then uploaded to the Basic Local Alignment Search Tool (BLAST; Johnson et al., 2008), an online application that searches for similar regions between biological sequences. For this study, BLASTP was used. Most algorithm settings were left as default, but low complexity regions were filtered. The results were downloaded and imported into Excel, where Ensembl and Flybase IDs and e-values were extracted and placed in a separate sheet before being exported as a tab-separated text file. Protein interaction values from both networks were obtained from STRING, imported into Excel and exported as tab-separated text files.

Data Visualisation

Graphs displaying the GSEA results were created using Microsoft Excel. Images of the networks were downloaded directly from the STRING application. Tables for gene

frequencies, centrality measures, and protein complex conservation scores were created using Microsoft Word.

Results

Gene Frequency Across All Studies

The initial screen of the literature identified 339 genes whose expression showed changes associated with AD pathology and were selected for study. Of these, 10 genes were found to be pseudogenes and were removed from further analyses, leaving a total of 329 genes from 52 papers (see appendix A for the complete list of papers). 179 genes had annotated changes in expression profiles, as indicated by fold difference values or authors' observations. Of these 179 genes, 93 were found to be upregulated in AD and 86 were downregulated. Expression patterns across genes with multiple instances were mostly consistent, with the exception of three genes: GRIA1 (downregulated in 2 studies, upregulated in 1), GRIA2 (upregulated in 2 studies, downregulated in 1), and PTK2B (upregulated in 1 study, downregulated in 1) these genes were not part of this study.

Following this analysis, the 10 most frequently identified genes with significant regulation changes, regardless of direction, in AD were selected for further study, and are shown in **Table 1**, along with selected functions of the proteins they encode. These functions were retrieved from the UniProt database.

Table 1. Most frequently identified genes with significant regulation changes in AD across all studies.

Rank	Human Gene	*Dros.* Ortholog	Functions (GO:BP)	Potential AD-Related Functions
1	Myc box-dependent-interacting protein 1/Amphiphysin II	Amph	Cytoskeleton organization, endocytosis, membrane organization	Tau protein binding, negative regulation of amyloid-beta function

	(BIN1)			
2	Phosphatidylinositol binding clathrin assembly protein (PICALM)	Lap	Endocytosis, vesicle-mediated transport, membrane organization	Tau protein binding, negative regulation of amyloid-beta function
3	ATP-binding cassette sub-family A member 7 (ABCA7)	-	Lipid transport, phagocytosis, cholesterol efflux	Negative regulation of amyloid-beta function, positive regulation of amyloid-beta clearance
4	Clusterin (CLU)	-	Chaperone binding, innate immune response, protein stabilization	Tau protein binding, negative/positive regulation of amyloid-beta formation, positive regulation of neurofibrillary tangle assembly
= 5	Complement receptor type 1 (CR1)	-	Negative regulation of complement activation, complement activation, classical pathway, negative regulation of interferon-gamma production	-
= 5	Myeloid cell surface antigen CD33 (CD33)	-	Cell-cell adhesion, regulation of immune response	-
= 5	MS4A6A	-	N/A – literature suggests involvement in signal transduction as part of multimeric receptor complex	-
= 6	Protein-tyrosine kinase 2-beta (PTK2B)	Fak	Regulation of actin cytoskeleton reorganization, regulation of cell adhesion, regulation of cell population proliferation, regulation of synaptic plasticity	Protein phosphorylation
= 6	Ephrin type-A receptor 1 (EPHA1)	Eph	Positive/negative regulation of cell migration, positive regulation of cell-matrix adhesion, cell surface receptor signalling pathway	Positive/negative regulation of kinase activity
= 6	Apolipoprotein E (APOE)	-	Cholesterol metabolic process, triglyceride metabolic process, lipoprotein biosynthetic process,	Tau protein binding, amyloid precursor protein metabolic process, positive/negative regulation of amyloid-beta

			positive/negative regulation of phospholipid efflux	formation, positive regulation of neurofibrillary tangle assembly, amyloid-beta binding
= 6	Sortilin-related receptor (SORL1)	-	Protein localization to Golgi apparatus, post-Golgi vesicle-mediated transport, positive regulation of ER to Golgi vesicle-mediated transport	Amyloid-beta binding, negative regulation of neurofibrillary tangle assembly, negative regulation of amyloid-beta formation

Frequently Identified Genes with Confirmed *Drosophila* Orthologs

To ascertain how these results can be applied to the development of *Drosophila* models expressing these genes, and subsequently determine how they relate to neurodegeneration, I identified the most frequently identified human genes with confirmed *Drosophila* orthologs. The 10 most frequently identified genes with *Drosophila* orthologs are shown in Table 2. Selected functions of their proteins are also presented; these annotations were retrieved from FlyBase and derived from the literature.

Table 2. Most frequently identified genes with significant regulation changes in AD with orthologues in flies and humans

Rank	Human Gene(s)	*Dros.* Ortholog	Primary Functions (GO:BP)	Expression Direction (▲/▼)
1	BIN1/Amphiphysin II	Amph	Cytoskeleton organization, membrane organization†	▼ (Dourlen et al., 2016; Bin. et al., 2019)
2	PICALM	Lap	Chemical synaptic transmission, clathrin-dependent synaptic vesicle endocytosis, synaptic vesicle transport	▲ (Bin. et al. (2019)
3	Glutamate receptors 1/2 (GRIA1/GRIA2)*	GluRIA	Ionotropic glutamate neurotransmitter receptor †	▲ (Aldred et al., 2012; McKeever et al., 2017) & ▼ (Ginsberg et al., 2012; Annese et al., 2018)

4	EPHA1/EPHA4	Eph	Axon guidance, regulation of cell population proliferation, pattern specification process †	▲ (Dourlen et al., 2016; McKeever et al., 2017)
5	PTK2B	Fak	Cell adhesion, cell migration, negative regulation of kinase signalling †	▲ (McKeever et al., 2017) & ▼ (Dourlen et al., 2016)
=6	Activity-regulated cytoskeleton-associated protein (ARC)	Arc2	Long-term memory, regulation of synaptic plasticity †	▲ (Aldred et al., 2012; Annese et al., 2018; Wes et al., 2015)
=6	Calcium/calmodulin-dependent protein kinase types IV/1D CAMK4/CAMK1D	CaMKI	Protein phosphorylation †	▼ (Annese et al., 2018)
=6	CD2-associated protein (CD2AP)	Cindr	Actin filament organization, receptor-mediated endocytosis, cytokinesis, actin cytoskeleton organization †	-
=6	Cation-independent mannose-6-phosphate receptor (IGF2R)	Lerp	Lysosomal transport, post-Golgi vesicle-mediated transport, lysosome organization †	-
=6	ATP-dependent 6-phosphofructokinase (PFKM/PFKL/PFKP)	Pfk	Glycolysis	▲ (Kelly et al., 2017) & ▼ (Puthiyedth et al. (2016)

* Multiple instances of GRIA1 and GRIA2 were found in the literature search, and both share GluRIA as their closest confirmed *Drosophila* ortholog; thus, for the purposes of this analysis, they were considered identical, as were multiple other genes sharing one *Drosophila* ortholog.

† See Appendix C for full list of references specific to this table.

Table 2 shows that only four genes from Table 1 (*BIN1*, *PICALM*, *EPHA1*, and *PTK2B*) have known *Drosophila* orthologs. Of these four genes, 3 (*PICALM*, *EPHA1*, and *PTK2B*) share similar functions with their *Drosophila* orthologs, suggesting they are evolutionary conserved. *BIN1* is also conserved although its ortholog *Amph* is involved in cytoskeleton and

membrane dynamics but apparently lacks its endocytic functions in *Drosophila* (Dräger et al., 2017).

Gene Frequency & *Drosophila* Orthologs - Discussion

Most of the genes in Table 1 can be linked to multiple biological processes, so discerning their products' absolute primary functions is not always feasible. Nevertheless, from Table 1 it is evident that these genes' proteins share similar functions, mainly in immunity, lipid metabolism, and endocytosis. Furthermore, 7 of the 10 genes are associated with processes related to AD pathology, particularly tau protein binding and amyloid-beta regulation.

The prominence of innate immunity as a common pathway is consistent with current literature suggesting chronic neuroinflammation as a key factor in AD pathology (Heneka et al., 2015), and there is a particular focus on the role of microglia in this process (Regen et al., 2017). One of the genes, *CD33*, encodes for a receptor protein expressed in microglia; increased expression of which is associated with greater AD pathology and reduced clearance of amyloid-beta 42 (Bradshaw et al., 2013; Walker et al., 2015). Furthermore, there is evidence that microglia may propagate tau pathology by phagocytosing tau aggregates and subsequently "seeding" them in other neurons via exosome secretion (Asai et al., 2015). Maphis et al. (2015) also observed increased tau pathology in *CX3CR1*-knockout mice, along with overexpression of *IL-1B* and *CD68* proteins. This is significant because these three genes, though not included in this analysis, were identified as significant from the wider literature search (see Appendix B).

Processes relating to lipid metabolism in relation to AD has mostly focused on *APOE*, of which the E4 allele is well-documented as a risk factor for developing AD (Michaelson et al., 2014). However, there is a growing body of research implicating lipid-related processes to

the development of NFTs, particularly in cholesterol metabolism and transport. This is exemplified by comparing AD with Niemann–Pick type C disease (NP-C), a rare genetic disorder characterised by impaired lipid transport and accumulation within neurons and subsequent neurodegeneration, including the formation of NFTs (Chang et al., 2005; Love, Bridges & Case, 1995). Immunohistochemistry experiments in Auer et al. (1995) found that NP-C NFTs were identical in composition to AD NFTs, while Sawamura et al. (2001) observed tau phosphorylation by mitogen-activated protein kinase (*MAPK*) activation, a protein noted for its role in phosphorylating tau in AD (Leugers, Koh, Hong & Lee, 2013). Interestingly, NFTs in NP-C have been noted to form closer to neurons containing high levels of cholesterol (Distl et al., 2003; Ghribi, Larsen, Schrag & Herman, 2006), suggesting that NFTs and tau phosphorylation may participate in a process reacting to the cholesterol.

Aberrations in genes involved endocytosis and other vesicle-related processes also lead to AD pathophysiology, including abnormal tau aggregations. *BIN1/Amphiphysin II*, the most frequently identified gene from the analysis, has a documented effect on tau-specific pathology (Chapuis et al., 2013; Lasorsa et al., 2018). The link between *BIN1*, tau, and endocytosis is unclear, but evidence suggests that *BIN1* may promote tau propagation by increasing endocytic flux, leading to internalisation of tau aggregates (Calafate, Flavin, Verstreken & Moechars, 2016), and subsequently releasing the tau via extracellular vesicles (Crotti et al., 2019). Additionally, underexpression of *PICALM*, the second most frequently identified genes and a crucial component of clathrin-mediated endocytosis, leads to increased levels of phosphorylated tau and NFTs (Ando et al., 2016; Ando et al., 2020).

In summary, this analysis highlighted four biological pathways – immunity, lipid metabolism, cytoskeletal organization, and vesicle endocytosis - involved in, or at least relevant to, the pathology of AD; an observation further supported by current literature suggesting that

further AD studies should focus on genes or proteins associated with these processes, with an emphasis on how they interact with each other.

Table 2 reveals that functions relating to immunity and lipid metabolism are virtually absent in the *Drosophila* orthologs. The absence of genes with immune-based functions might be expected, given the significant differences between mammalian and arthropod immune systems (Sheehan, Garvey, Croke, & Kavanagh, 2018). However, lipid metabolism is an important physiological process in *Drosophila*, and there is evidence of neurodegeneration in *Drosophila* resulting from the loss of the gene *Npc1a*, which is involved in cholesterol metabolism (Phillips, Woodruff, Liang, Patten & Broadie, 2008). Why more genes involved in lipid metabolism are not present is unclear. Nevertheless, functions relating to endocytosis and cytoskeletal dynamics are strongly represented in Table 2 and comparable to the mammalian homologues in Table 1. Another feature of the data from *Drosophila* is the significant incidence of genes with roles in synaptic processes and signalling pathways. There is evidence to suggest that these processes interact considerably, and that aberrations in one process has a knock-on effect on the others. Ojelade et al. (2019) found that *cindr,* one of the genes identified in this analysis, is localised to synaptic terminals, and absence of this protein impairs both synaptic vesicle recycling and synaptic plasticity by disrupting the ubiquitin-proteasome system (UPS); in turn, this increases the levels of plasma membrane calcium ATPase (PMCA) and synapsin, resulting in the observed dysfunctions. Additionally, disruptions in the *Drosophila* protein *Futsch* (an ortholog of the human gene MAP1B, identified in the literature search but not included in this analysis) led to microtubule and axonal transport defects, resulting in synaptic defects (da Cruz et al., 2005), while loss of the *Drosophila* gene *Vps35* inhibited endocytic processes, caused synaptic signalling defects, and affected cytoskeleton organisation (Korolchuk et al., 2007).

This "cause and effect" also extends to *Drosophila* models of AD pathology. For example, Blard et al. (2007) found that four proteins (*cheerio*, *Chd64*, *jaguar*, and *Paxillin*), which interact with the cytoskeleton directly, modulated tau pathology in a mutant *Drosophila* model, consequently leading to synaptic dysfunction. Yu et al. (2020) observed the effects of the PICALM ortholog *lap*, another gene identified in this analysis, on $A\beta_{42}$ toxicity in a *Drosophila* model of AD. Overexpression of *lap* ameliorated Aβ42 toxicity and reduced levels of presynaptic vesicular glutamate transporter (VGlut); this is important because accumulation of VGlut impairs synaptic transmission. Secondly, *lap* affected *Amph* localisation postsynaptically and *Amph*, in turn, regulated the localisation of *GluRII* receptors. Overall, it could be suggested that both proteins act in a cascading manner to prevent $A\beta_{42}$-induced dysfunctions in synaptic transmission and vesicle transportation, and that aberrances in either their levels or structures may enhance AD pathology.

To conclude, this analysis has demonstrated that, in *Drosophila*, two processes – endocytosis and cytoskeletal organization – emerge as being particularly relevant to the pathology of AD in both humans and *Drosophila*, suggesting that further research should focus on the functions of the identified genes in relation to these biological pathways to determine how abnormalities in their encoded products contribute to neurodegenerative processes.

Functional Enrichment Analysis

To determine the roles of genes identified in the study, genes were subjected to gene ontology (GO) functional enrichment analyses. GO enrichment analyses use standardised annotations of gene products derived from manually curated databases and works by comparing the frequency of different annotation terms associated with listed inputs. This is a relatively powerful way of identifying the physiological roles most frequently associated with the genes

under analysis (Maleki et al., 2020). The analysis typically focuses on three aspect of gene product function:

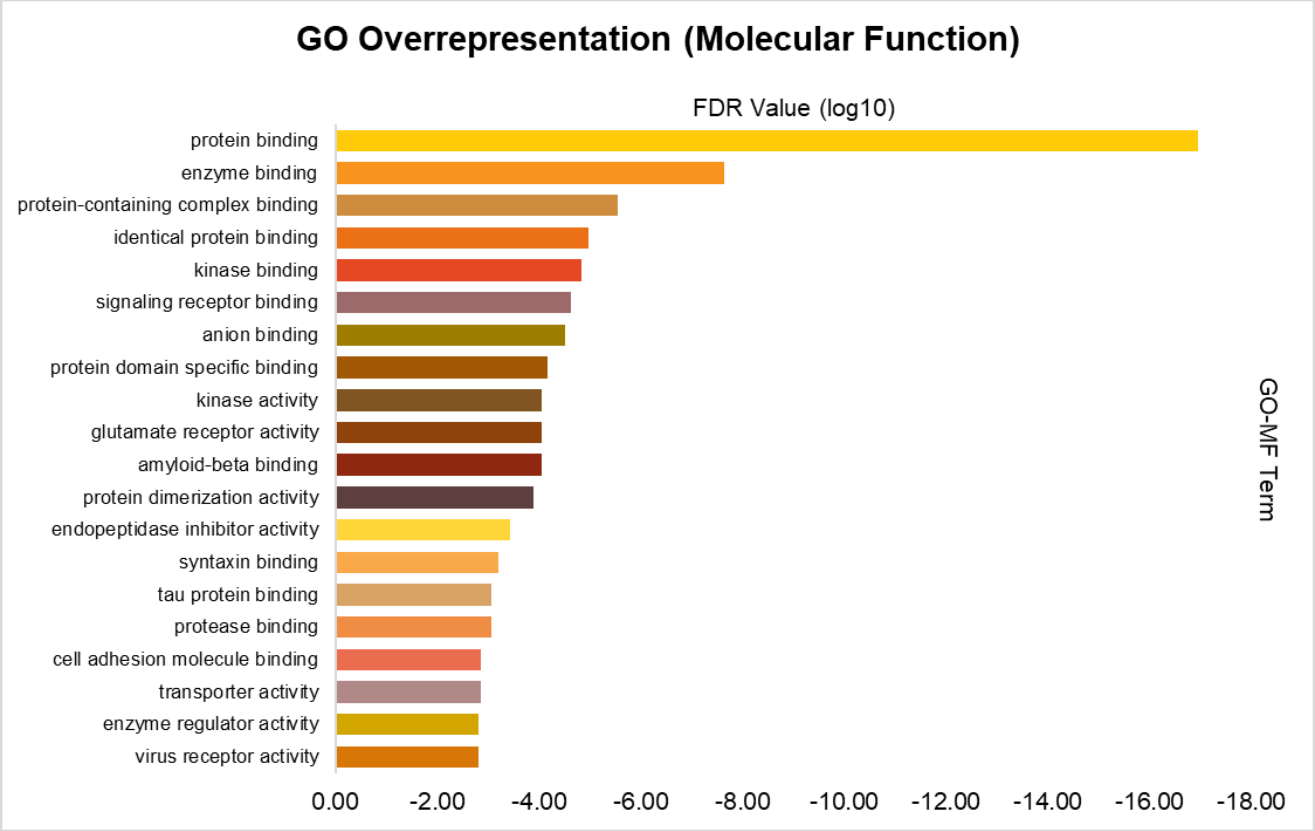
1) Molecular Function – activities undertaken by gene products at the molecular level, such as *catalytic activity* or *protein binding*.
2) Biological Process – multiple, sequential molecular events that together form a larger process, such as *DNA repair* or *synaptic signalling*.
3) Cellular Component – cellular or macromolecule locations where gene products perform their functions, such as the *nucleus* or the *ribosome*.

I elected to undertake an analysis of the gene data for each GO category. For each term discussed in the text below, the number of genes associated with that term is represented by *n*, while significance of a term is represented by *p*.

Molecular Function of Human Genes (GO:MF)

The Top 20 outcomes of the GO:MF analysis of the human genes are shown in Figure 2. This shows that terms relating to "binding" are heavily represented, with "protein binding" being markedly more enriched compared to other terms ($n = 194$, $p = 1.11\times10^{-17}$), followed by "enzyme binding" ($n = 79$, $p = 2.28\times10^{-8}$), "protein-containing complex binding" ($n = 43$, $p = 2.80\times10^{-6}$), "identical protein binding" ($n = 61$, $p = 1.09\times10^{-5}$), and "kinase binding" ($n = 33$, $p = 1.47\times10^{-5}$).

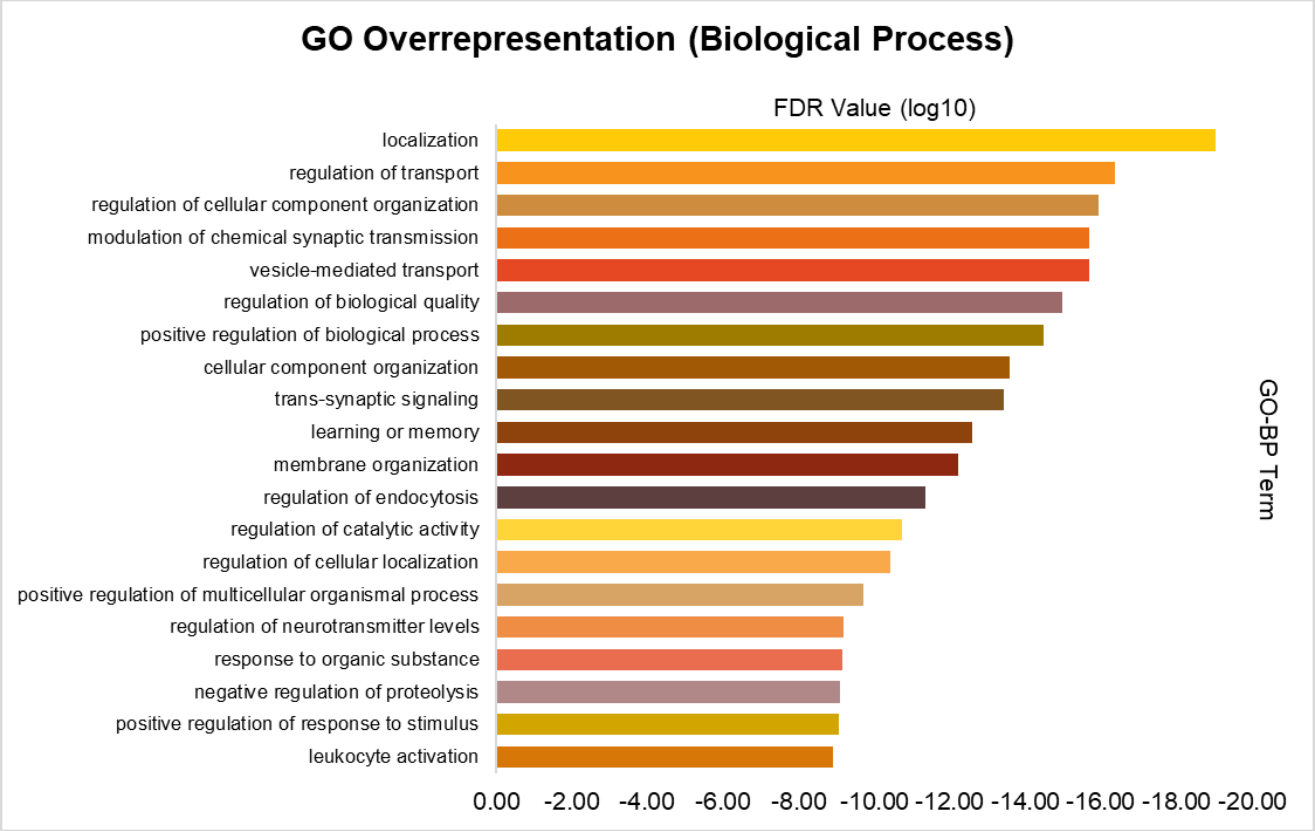
Figure 2. Top 20 enriched GO:MF annotations for human genes dataset.



Biological Processes of Human Genes (GO:BP)

Biological Process analysis demonstrates a more diverse number of GO:BP terms enriched in the human gene dataset (Fig 2), and may be more informative. The enriched terms mainly relate to synaptic physiology, endocytic functions, cellular organization, and regulatory processes. “Localization” is the most enriched term (n = 174, $p = 9.19\times10^{-20}$), followed by “regulation of transport” (n = 87, $p = 4.39\times10^{-17}$), “regulation of cellular component organization” (n = 101, $p = 1.19\times10^{-16}$), “modulation of chemical synaptic transmission” (n = 37, $p = 2.07\times10^{-16}$), and “vesicle-mediated transport” (n = 84, $p = 2.08\times10^{-16}$).

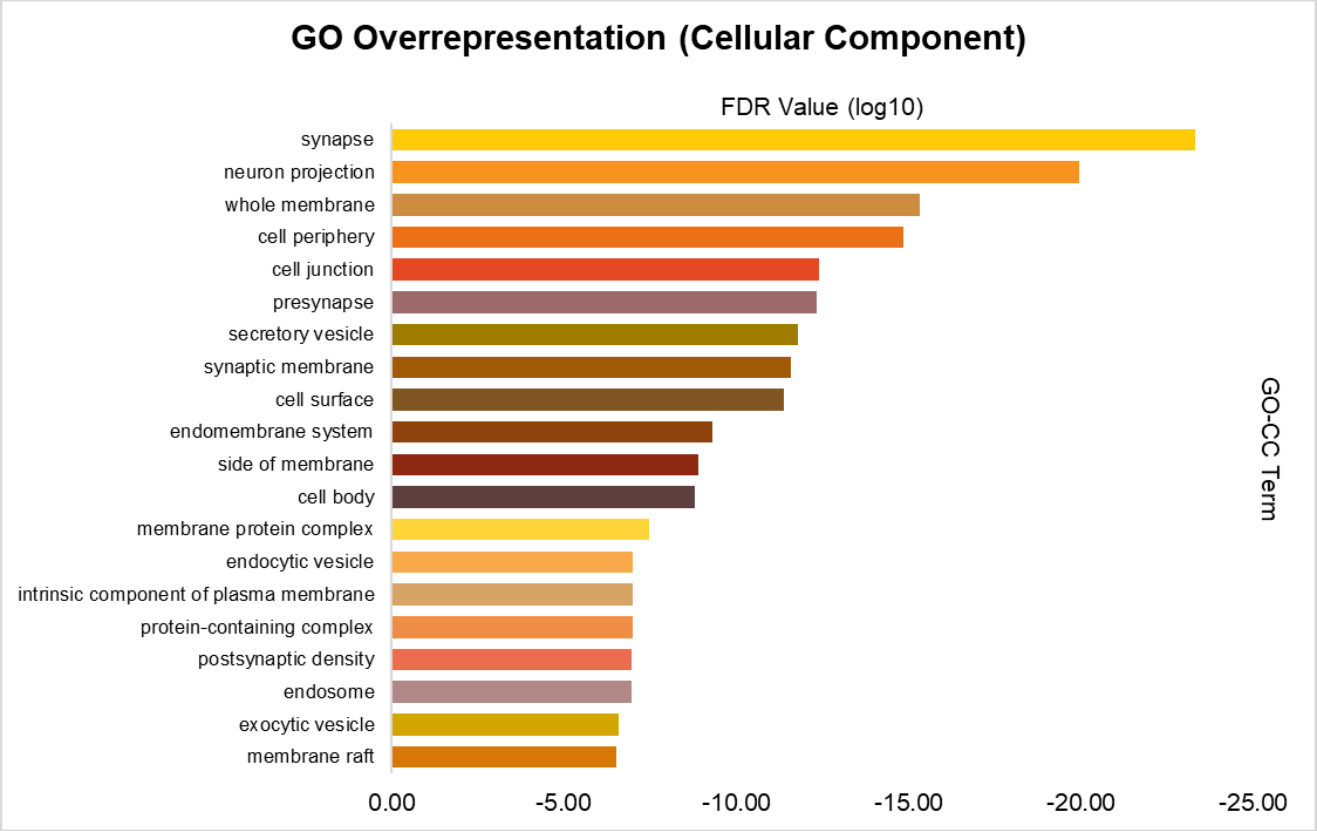
Figure 3. Top 20 enriched GO:BP annotations for human genes dataset.



Cellular Components of Human Genes (GO:CC)

This analysis is perhaps the most straightforward and informative, with most terms relating to either neuronal substructures, cellular membranes, or endocytic macromolecules. As Figure 4 shows, “synapse” is the most enriched term (n = 69, p = 4.98×10^{-24}), followed by “neuron projection” (n = 74, p = 1.16×10^{-20}), “whole membrane” (n = 78, p = 4.90×10^{-16}), “cell periphery” (n = 161, p = 1.47×10^{-15}), and “cell junction” (n = 56, p = 3.84×10^{-13}).

Figure 4. Top 20 enriched GO:CC annotations for human genes dataset.



Human Functional Enrichment Analysis - Discussion

The term "protein binding" (GO:0005515) is defined by GO as "*Interacting selectively and non-covalently with any protein or protein complex (a complex of two or more proteins that may include other nonprotein molecules)*". It is a broad category covering numerous, more specific terms related to binding (e.g. "clathrin binding"), so its significance may be biased, and too general to be meaningful. Nevertheless, binding functions are prevalent in this analysis, so its significance may simply reflect this. This also seems to be true for "enzyme binding" (GO:0019899), another term that covers functions relating to enzyme interactions (e.g. "lipase binding").

"Kinase binding" is the most enriched, specific term (n = 33, $p = 1.47 \times 10^{-5}$) and reflects the role of kinases in the pathophysiology of AD. Kinases are enzymes that catalyse the process of phosphorylation, proteins that bind to kinases therefore affect their activities, and

perturbations in these proteins can have wider biological consequences. For example, elevated levels of death-associated protein kinase (*DAPK*) aberrantly activate the kinases MARK1/2, which in turn destabilise microtubules by phosphorylating tau, subsequently leading to tau toxicity (Wu et al, 2011). Furthermore, deficiencies of the TREM2 protein exacerbates tau pathology by hyperactivation of tau kinases (Jiang et al., 2015; Bemiller et al, 2017).

Aberrations in kinases themselves also contribute significantly to AD pathology and is reflected by enrichment of the term "kinase activity". Glycogen synthase kinase 3β (*GSK3β*) is a kinase heavily implicated in the development and progression of AD. Normally, *GSK3β* directly phosphorylates tau protein by binding phosphoryl groups to the amino acids serine and threonine (Flaherty, Soria, Tomasiewicz, and Wood, 2000; Cho and Johnson, 2004). However, in AD, *GSK3β* is overactivated leading to hyperphosphorylation of tau contributing to the pathology of AD (Rankin, Sun, and Gamblin, 2007; Wang, Grundke-Iqbal, and Iqbal, 2007).

Like the GO:MF term "protein binding", "localization" (GO:0051179) is an umbrella term covering many more specific terms (e.g. "macromolecule localization"), so its enrichment may be skewed; this also applies to the term "regulation of transport" (GO:0051049). Both these terms may be too general from which to form conclusions.

However, there are more diverse terms than in GO:MF analysis, many of which relate to functions identified in the frequency analysis. For example, terms relating to synaptic processes ("modulation of chemical synaptic transmission", "trans-synaptic signalling", "regulation of neurotransmitter levels") are significantly enriched, and there is evidence that demonstrates the effects of tau-mediated pathology in these areas. For example, in mice, Hoover et al (2010) found that mislocalised tau in dendritic spines disrupts synaptic function

via impairment of several AMPA and NMDA receptors, including *GluR1* and *GluR2* (the products of the genes *GRIA1* and *GRIA2*, respectively). Additionally, *EAAC1*, the product of the gene *SLC1A1* and a glutamate transporter have abnormal accumulation in hippocampal CA2-CA3 pyramidal neurons in AD patients that contained significant levels of abnormal tau (Duerson et al., 2009).

The enrichment of terms relating to vesicle-related functions ("vesicle-mediated transport" and "regulation of endocytosis") supports the results of the frequency analysis and is consistent with current literature. The propagation of tau by exosome secretion has been discussed and there is further evidence suggesting pathogenic tau species are internalised by endocytosis, leading to intracellular accumulation (Wu et al., 2013). Furthermore, pathogenic tau appears to bind to synaptic vesicles, resulting in various presynaptic impairments (Zhou et al., 2017). Regarding specific genes, polymorphisms in the vesicle-associated membrane protein 1 (*VAMP1*) gene, which mediates both Ca^{+}-triggered synaptic-vesicle exocytosis and endocytosis (Deák et al., 2004; Deák, Shin, Kavalali, and Südhof, 2006), were associated with varying levels of AD pathology by affecting the secretion of Aβ (Sevlever et al., 2015). Synaptophysin (*SYP*) binds cholesterol in synaptic vesicles and suggested to contribute to the separation of microvesicles from the membrane (Thiele, Hannah, Fahrenholz, and Huttner, 2000). *Syp* expression in AD-affected tissues is markedly decreased (Heinonen et al., 1995; Callahan, Vaules, and Coleman, 2002), and has been correlated with clinical cognitive decline (Sze et al., 1997).

As highlighted by the other analyses, numerous genes and their products are localised at the synapse, especially presynaptically, and there is a wide body of evidence that highlights synaptic dysfunction in AD pathophysiology. Synaptic loss is evident in most of the brain

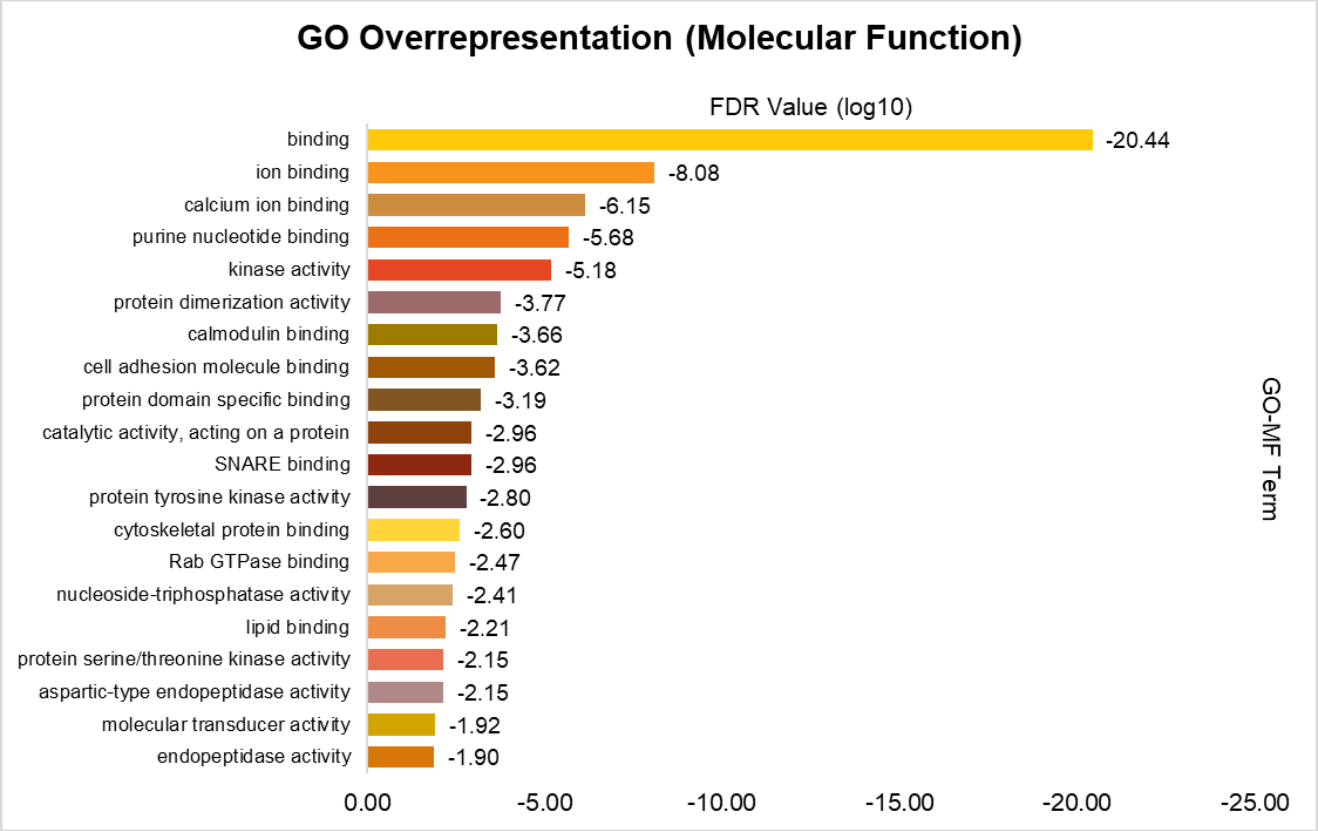
over the course of AD, including in the frontal and temporal lobes (DeKosky and Scheff, 1990; Scheff and Price, 1993), but is especially prevalent in the hippocampal region. In fact, synaptic loss appears to be an early feature of AD, correlating with cognitive decline (Scheff, Price, Schmitt, and Mufson, 2006; Scheff, et al., 2007). Synaptic density in the dentate gyrus in AD patients is particularly decreased compared to controls (Scheff, Sparks, and Price, 1996; Scheff and Price, 1998).

Other enriched terms in this analysis correlate to genes that encode for membrane receptors (for example, GRIA1) or genes that regulate and/or participate in endocytosis, exocytosis, and other vesicle-related functions.

Drosophila GO:MF

Figure 5 demonstrates that, like the human GO:MF analysis, terms related to binding are overrepresented in the *Drosophila* genes data, with the term "binding" itself more significant than any other term (n = 179, $p = 3.65\times10^{-21}$). This is followed by "ion binding" (n = 79, $p = 8.30\times10^{-9}$), "calcium ion binding" (n = 20, $p = 7.08\times10^{-7}$), "purine protein binding" (n = 40, $p = 2.11\times10^{-6}$), and "kinase activity" (n = 23, $p = 2.11\times10^{-6}$).

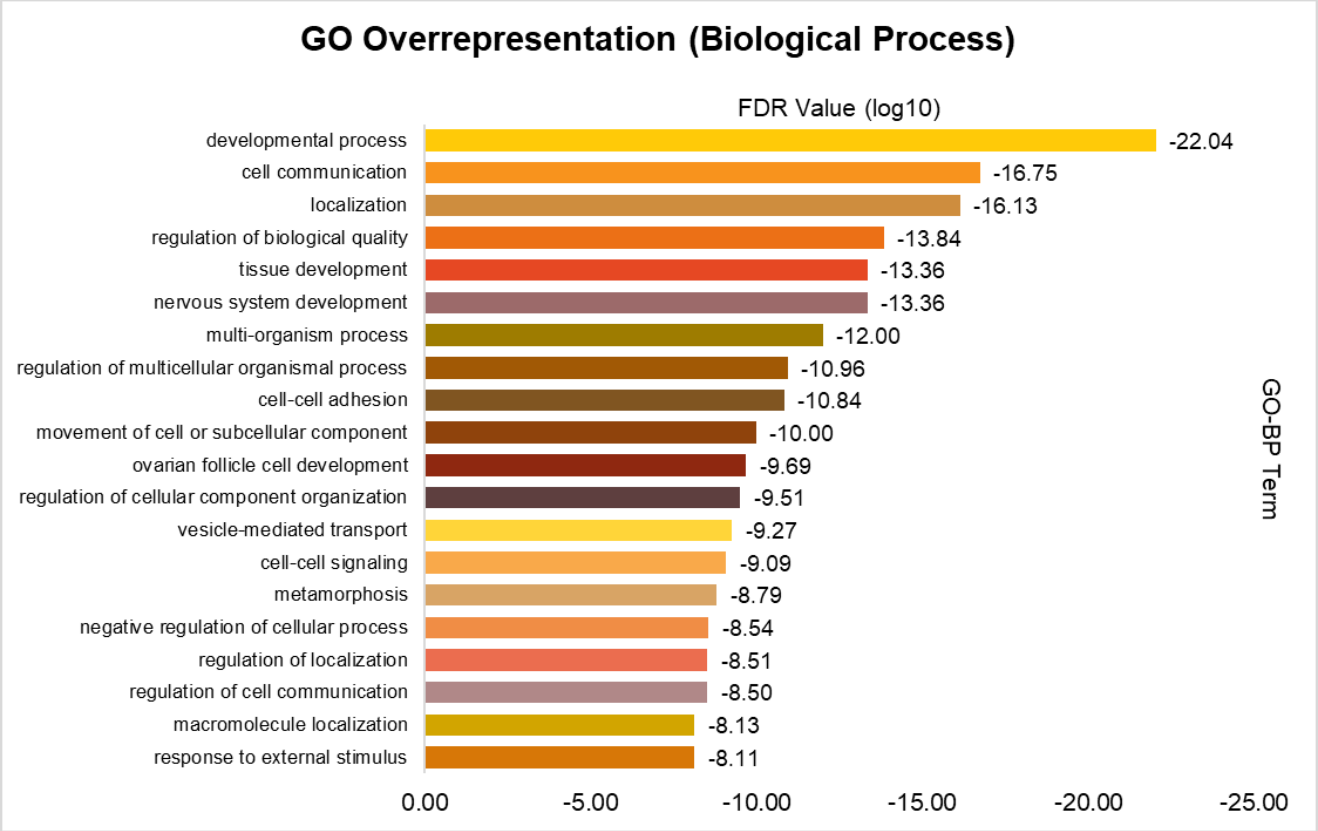
Figure 5. Top 20 enriched GO:MF annotations for Drosophila genes dataset.



Drosophila GO:BP

Like to the GO:BP analysis for human genes, Figure 6 demonstrates a wide range of enriched terms for *Drosophila*. Both analyses also share terms linked to vesicle- and regulatory-related functions. However, terms covering development are the most enriched, with "developmental process" first (n = 128, $p = 9.11\times10^{-23}$), followed by "cell communication" (n = 83, $p = 1.77\times10^{-17}$), "localization" (n = 100, $p = 7.45\times10^{-17}$), "regulation of biological quality" (n = 71, $p = 1.45\times10^{-14}$), and "tissue development" and "nervous system development" (n = 62, $p = 4.36\times10^{-14}$ for both).

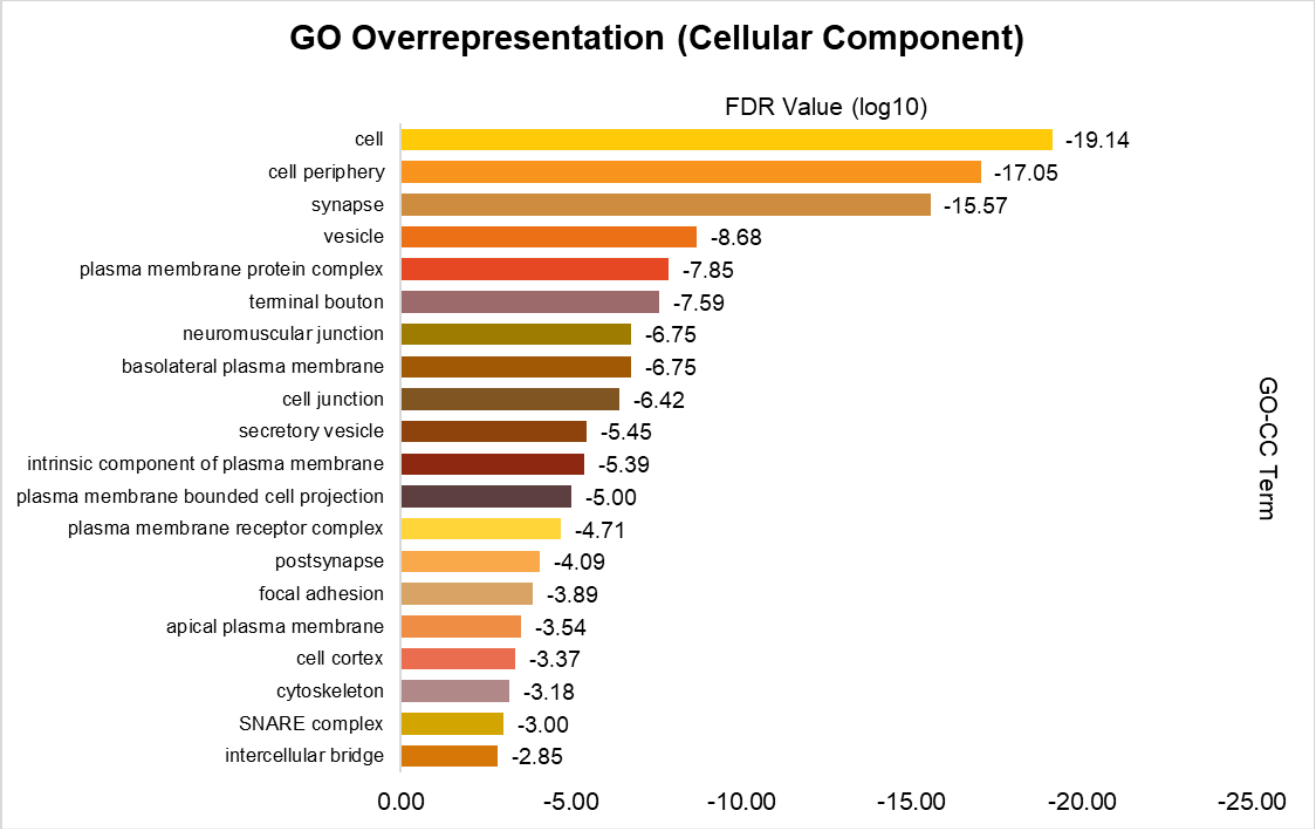
Figure 6.. Top 20 enriched GO:BP annotations for Drosophila genes dataset.



Drosophila GO:CC

Like the human gene analysis, this is arguably the most elementary, with many terms relating to nervous system structures and cellular membranes. Figure 7 shows, “cell” is the most enriched term (n = 203, p = 7.23×10^{-20}), followed by “cell periphery” (n = 75, p = 8.85×10^{-18}), “synapse” (n = 33, p = 2.70×10^{-16}), “vesicle” (n = 365, p = 2.08×10^{-9}), and “plasma membrane protein complex” (n = 19, p = 1.41×10^{-8}).

Figure 7. Top 20 enriched GO:BP annotations for Drosophila genes dataset.



Drosophila Functional Enrichment Analysis - Discussion

Like the term "protein binding" from the human GO:MF analysis, "binding" is a comprehensive term with numerous, more specific derivative terms, and may be far too broad in scope to draw any meaning conclusions. Conversely, and like "protein binding", its significance may be interpreted as a general reflection of other enriched binding terms present in this analysis.

"Calcium ion binding" is the most enriched *specific* term here, and research on *Drosophila* models of AD and other tauopathies have demonstrated the role of calcium in driving pathological processes. For example, overexpression of *CamKII*, the *Drosophila* ortholog of calcium/calmodulin-dependent protein kinase type II delta chain (*CAMK2D*) and a paralog of *CamKI*, promotes neurodegeneration caused by phosphorylated tau (Oka et al., 2017). Using

a transgenic *Drosophila* model, Michno et al. (2009) found that *psn* (*Drosophila* ortholog of presenilin 1 (PSEN1) caused deficits in intracellular calcium stores; these decrements were ameliorated by *Cam*, the ortholog of *CALM3*.

“Kinase activity" another highly enriched term ($n = 21$, $p = 6.54\times10^{-6}$), and one of the few identified in both species’ GO:MF analyses, highlighting the crucial role kinases in the pathology of AD. Like its human ortholog *GSK3β*, overexpression of *Shaggy* (*sgg)* exacerbates tau pathology *in vivo via* abnormal tau phosphorylation (Jackson et al., 2002; Mudher et al., 2004). Conversely, sgg inhibition reduces both tau and Aβ pathology (Sofola et al., 2010; Sarkar, 2021). *Par-1*, an ortholog of *MARK1*, hyperphosphorylates tau when overexpressed (Ando et al., 2016), while its inhibition reduces tau phosphorylation (Iijima-Ando et al., 2012). Interestingly, like their human orthologs, *par-1* and *sgg* directly interact, with the former regulating the latter where reduced *par-1* expression results in increased *sgg* (Ando et al., 2016).

Considering AD is a neurodegenerative disorder typically occurring later in life, the significant enrichment of the term “developmental process” might seem unexpected, but there is evidence suggesting involvement of development genes and/or pathways in AD pathology. Studies have found a relationship between presenilins and *Notch*, a membrane receptor crucial in many developmental processes (Artavanis-Tsakonas et al., 1999). *PSEN1* and *PSEN2* mediate the release of the cytoplasmic domain of Notch and its subsequent localisation to the nucleus (Steiner et al., 1999; Stralh and Greenwald, 2001) and misexpression of presenilin negatively affect Notch signalling, and leads to AD-like pathology (Song et al., 1999; Ye, Lukinova, and Fortini, 1999)

“Vesicle-mediated transport” ($n = 35$, $p = 5.41\times10^{-10}$) is enriched in both GO:BP analyses, supporting its significance in AD pathology across species. Both synaptic exo-/endo-cytosis

and axonal transportation are disrupted by overexpression of tau, (Chee et al., 2005; Talmat-Amar, Arribat, and Parmentier, 2018). A loss-of-function mutation in the APP ortholog *Appl* results in endolysosomal dysfunction in neurons, eventually leading to cellular death and aggregation (Kessissoglou et al., 2020).

"Cell" is too broad a term for any meaningful interpretation. The enrichment of the terms: "synapse", "neuromuscular junction", and "postsynapse" and "vesicle" and "secretory vesicle" lend further support to the notion of AD primarily affecting synaptic processes and vesicle-related functions, respectively.

Protein-Protein Interaction Network (PPIN) Analysis

The results presented above show that some proteins and biological pathways are more prominent than others in AD. More specifically, they have highlighted the prominence of *BIN1*/*Amph* and endocytic functions in both human and *Drosophila* models of AD. However, they have further illustrated AD as an intrinsically complex disease that cannot be reduced to a single protein or biological pathway; rather, it is a sum of molecular interactions that give rise to emergent biological processes, which in turn lead to the complex phenotype of AD.

A protein-protein interaction network (PPIN) uses graph theory to illustrate these interactions, whereby proteins are represented by nodes and their interactions by edges. By rendering PPINs as discrete mathematical objects, various topological analyses can be performed on them, such as centrality analysis and clustering analysis. In turn, these methods can be used to infer wider biological properties of a system, such as identifying functionally related modules (Pereira-Leal et al., 2003; Wu et al. 2009), elucidating disease mechanisms (Xu et al., 2006; Luo et al., 2015), and isolating conserved interactions between species (Sharan et al., 2005). The latter point is of particular interest here, as finding protein

interactions that occur in AD in both humans and *Drosophila* models would aid in clarifying possible, universal mechanisms of AD pathology.

Identified genes were inputted into the STRING application to generate the human network. For *Drosophila*, genes were converted to their fly orthologs before being inputted into STRING. These networks were imported into Cytoscape for further analyses.

Human Protein-Protein Interaction Network

Due to its size, the image of the human network can be found in Appendix D336 genes were entered into STRING, and of these, 328 were recognised to have recorded proteins. Deletion of unconnected proteins left a total of 306 nodes, with 2007 edges (interactions). Table 3 shows the average values of network-wide measures.

Table 3. Average human network-wide statistics.

Network Measure	Average Value
Average No. of Neighbours	13.118
Network Diameter	7
Network Radius	4
Characteristic Path Length	2.824
Clustering Coefficient	0.366
Network Density	0.043
Network Heterogeneity	0.976

Network Centralization	0.208

Before continuing, a discussion on network-wide topological properties is warranted. Within network science literature, networks are claimed to be different from random networks if they meet the criteria of certain, universal "laws": the *scale-free* law and the *small-world* law. Networks are *scale-free* if their degree distribution (the fraction of nodes with a specific degree value; $P(k)$) follows a power law, and they are considered *small world* if they have high average *clustering coefficients* (the tendency of nodes to form highly connected modules) and low *average path lengths* (average number of steps required to connect every pair of nodes through their shortest paths). Both laws are ubiquitously present in network science research, where networks are considered valid only if they satisfy the laws.

Recently the validity of these laws has been questioned on both theoretical and empirical grounds. Mendez and Helden (2009) provide a comprehensive review on the most common "myths" attributed to the two laws. Briefly, they find that power law interpretations stem from graphical misrepresentation, where the degree and probabilities of a real network are plotted using logarithmic scales while random networks are plotted linearly; that the term "scale-free" is often ill-defined in many papers; that small-worldliness may be falsely conferred to a network via irrelevant shortcuts; and that the small-world law may be more relevant to signalling pathway networks than PPINs.

Of the two laws, scale-freeness has come under particular scrutiny. Employing various on a wide range of real-world networks, Broido and Clauset (2019) found that empirically scale-free networks are rare, with only 6% of biological networks meeting their criteria, most of which are metabolic networks. Smith, Kim, and Walker (2021) repeat these results in biochemical networks, finding very few networks that are more than super-weakly scale-free.

There is less critical literature on the small-world property, but Telesford, Joyce, Hayasaka, Burdette, and Laurienti (2011) note that the criteria for small-wordliness is not strict, and that small-world networks are probably overestimated.

Considering this research, it would be unproductive to determine whether the human and *Drosophila* networks follow these laws. However, for posterity and to demonstrate consistency with at least one law, I will determine whether the networks follow the small world property.

Small World Property of Human AD Network

The small-world property for the human AD network was calculated according to the equation proposed by Humphries, Gurney, and Prescott (2006):

$$\gamma^i = \frac{C_c}{C_r}, \lambda^i = \frac{L_c}{L_r} \;\&\; S^1 = \frac{\gamma^i}{\lambda^i}$$

C_c and C_r represent the clustering coefficients of the real and random networks, respectively, while L_c and L_r represent the average path lengths of the real and random networks, respectively. To fulfil the small-world criteria, $\gamma^i > 1$ and $S^i > 1$. The results show that $\gamma^i = 8.5$ and $S^i > 7.56$; thus, the human AD network meets the criteria for the small-world law.

Global Network Properties of Human Protein-Protein Interaction Network

In global network analysis, the most important measures are arguably *average number of neighbours*, *clustering coefficient*, and *characteristic path* length. *Average No. of Neighbours* refers to the average connectivity of the nodes in the network, and as Table 3 shows this average is relatively high (13.118). In other words, proteins within the network have a higher-

than-average number of interactions with one another. This high level of connectivity may reflect the complexity of AD as a pathology.

Clustering coefficient, measures the tendency of nodes to form highly connected subgraphs, or modules. With a value of 0.366, it appears that proteins in this network do form modules, and a later analysis on protein complex conservation will demonstrate the significance of these modules.

Characteristic path length (CPL) refers to the average number of steps required to connect every pair of nodes through their shortest paths. Using Xu, Bezakova, Bunimovich, & Yi (2011) as a reference point, this network has a relatively low CPL compared to the observed human average (4.39). This indicates that the network displays a small-world property.

Comparison of AD Human Network to Random Networks

In theory, the constructed AD network should be significantly different from a randomly generated network; however, there is a possibility this is not the case. Using the method of Sun & Zhaoo (2010), the human AD network was compared to random networks. Using the R programming language in RStudio (RStudio Team, 2020), specifically the igraph package (Csardi & Nepusz, 2006), 1000 Erdős–Rényi model networks were generated and subjected to the following analyses: degree (K), betweenness (BC), clustering coefficient (TRNS). From these analyses, 307 values (equal to the number of values from the human AD network) were randomly sampled and compared to the human AD network using the Wilcoxon signed-rank test. The full code can be found in Appendix E.

Significant differences were found between the AD network centralities and the random network centralities. Both AD degree and betweenness centralities were significantly different ($p < 0.01$) from random, while clustering coefficient was exponentially different (p

$< 2.2\times10^{-16}$); when adjusted to account for calculation differences, the results were still significant ($p < 0.01$). Overall, this shows that the AD human network is distinct from random.

Human AD Network Centrality Analyses

To identify the proteins most central to the network, four measures of centrality were conducted with the Centiscape plugin: degree, closeness, betweenness, and eigenvector. Degree refers to the number of edges of a particular node. The higher the degree, the more central the node is to the network. Closeness centrality (CC) is the average shortest distance from one node to every other node within the network, and like degree, denotes how central a given node is in a network. Betweenness centrality (BC) measures the frequency in which a given node appears on the shortest pathways between two nodes; high betweenness nodes are regarded as highly influential because they can dictate the flow of information within a network. Eigenvector (EV) centrality measures the influence of a node within a network. Table 4 details the full results for degree, closeness, betweenness, and eigenvector for the top 15 proteins, ordered by degree.

Table 4. The fifteen proteins with consistently high values across all measures in network centrality analyses. Bold denotes proteins that are within the top five for two or more measures.

Protein	Degree (*k*)	Closeness (CC)	Betweenness	Eigenvector
APP	**76**	**0.001692**	**6408.640**	**0.2387**
SRC	**73**	**0.001686**	**9257.077**	**0.1886**
APOE	**63**	**0.001605**	**4045.472**	**0.2202**

TNF	**62**	**0.001623**	**6168.808**	**0.1650**
EGFR	**61**	**0.001639**	**7529.401**	**0.1580**
BDNF	58	0.001587	5073.210	0.1579
ACTB	46	0.001600	5557.391	0.1146
CLU	45	0.001490	1541.986	0.1691
GFAP	44	0.001517	2353.763	0.1392
DLG4	44	0.001493	3108.462	0.1155
PICALM	40	0.001433	1853.929	0.1481
BIN1	40	0.001416	1012.724	0.1475
SYP	40	0.001484	2158.747	0.1176
GRIA1	40	0.001437	1911.741	0.0934
PSEN1	39	0.001502	1452.863	0.1488

As Table 4 demonstrates each measure consistently features the proteins *APP*, *SRC*, *APOE*, *TNF*, and *EGFR*; of these, *APP* and *SRC* always occupied the top five. The degree and betweenness centralities are of particular interest. Yu et al. (2007) classified proteins in a given network depending on their degree and betweenness, as illustrated by Table 5 below.

Table 5. Classification of proteins according to their k/BC values. Adapted from Yu et al (2007).

	Low Betweenness	High Betweenness
Low Degree	Nonhub-nonbottleneck (NH-NB)	Nonhub-bottleneck (NH-B)
High Degree	Hub-nonbottleneck (H-NB)	Hub-bottleneck (HB)

To identify proteins from each category, the averages of the centralities plus one or two standard deviations of the mean were used as cutoff values. These values are displayed in Table 6 below.

Table 6. Cutoff values for identifying proteins in each category. Degree values are rounded to their nearest whole number.

	Degree	Betweenness
Mean ($\bar{x}$)	13	557.857
$\bar{x}$ + 1 SD	26	1639.920
$\bar{x}$ + 2 SDs	39	2721.984

Human Hub-Bottlenecks (HBs)

Using the $\bar{x}$ + 2 SDs cutoff values, the proteins *APP*, *SRC*, *APOE*, *TNF*, *EGFR*, *BDNF*, *ACTB*, and *DLG4* from Table 4 can be categorised as hub-bottlenecks (HBs). Yu et al. (2007) found that HBs correspond to highly centralised proteins that constitute parts of signal transduction pathways, and that they act as connectors for protein complexes within a network. This sentiment is supported by Chandramohan, Kiran, and Nagarajaram (2021, preprint), who also found that mixed (MX)/HB proteins are enriched in GO processes relating to signalling and metabolic pathways compared to other protein types.

Cursory GO analyses of these eight genes show significant enrichment of terms related to these pathways, particularly regarding protein kinases. Significantly enriched GO:BP terms relating to protein kinases include "regulation of protein kinase activity" (n = 8, p = 1.43×10^{-8}), "regulation of protein serine/threonine kinase activity" (n = 6, p = 1.13×10^{-6}), "positive regulation of protein kinase activity" (n = 6, p = 1.38×10^{-6}), "regulation of peptidyl-tyrosine phosphorylation" (n = 5, p = 1.74×10^{-6}), and "regulation of MAP kinase activity" (n = 5, p = 6.55×10^{-6}).

Human Nonhub-Bottlenecks (NH-Bs)

For this study, proteins are classed as NH-Bs if their degree and betweenness values are below and above the $\bar{x}$ + 1 SD cutoff, respectively. Six proteins can be categorised as NH-Bs: elongation factor 1-alpha 1 (*EEF1A1*; k = 26, *BC* = 2372.6810), polypyrimidine tract-binding protein 1 (*PTBP1*; k = 18, *BC* = 2252.293), heterogeneous nuclear ribonucleoprotein K (*HNRNPK*; *k* = 20, *BC* = 2012.249), beclin-1 (*BECN1*; k = 20, *BC* = 1934.165), histone H2A.Z (*H2AFZ*; *k* = 15, *BC* = 1923.636), and superoxide dismutase 2, mitochondrial (*SOD2*; *k* = 23, *BC* = 1654.314).

According to Yu et al. (2007), NH-Bs are important in maintaining overall network topology, with their removal resulting in network partitioning. They also control the flow of information within a network; however, this property mainly applies to directed regulatory networks, and has less relevance to an undirected interaction network such as this one. Regardless of network type, they appear to connect functionally similar protein complexes. Chandramohan and colleagues (2021, preprint) found that NH-Bs (referred to as PBs in their study) are enriched in GO:BP terms relating to immunity, transport, and metabolism, and are more likely to be involved in signal transduction pathways.

This is supported by a GO analysis of the six cited proteins, though there is less consistency among the terms compared to those found for the MX proteins. A few terms that can be inferred as relating to immunity are significantly enriched, including “cellular response to endogenous stimulus” (*n* = 4, *p* = 0.0114), “cellular response to stimulus” (*n* = 6, *p* = 0.0244), and “cellular response to toxic substance” (*n* = 2, *p* = 0.0299). Interestingly, terms concerning RNA-specific processes are also significantly enriched, including “negative regulation of mRNA splicing, via spliceosome” (*n* = 2, *p* = 0.0112), “regulation of RNA metabolic process” (*n* = 5, *p* = 0.0302), and “mRNA splicing, via spliceosome” (*n* = 2, *p* = 0.0434). This is interesting because isoforms of tau are produced via the alternative mRNA splicing of

exons 2, 3, and 10 of the gene. Proteins involved in this process that are abnormally expressed in AD could lead to fundamental deficits in tau protein itself. Alternatively, they could affect other proteins produced by alternative splicing, which in turn interact with or influence tau.

Human Hub-Nonbottlenecks (H-NBs)

H-NBs are the opposite of NH-Bs, with high degree values but low betweenness values. Again, the $\bar{x}$ + 1 SD cutoff was used to identify H-NBs, resulting in 24 proteins being identified as H-NBs. To discuss each protein individually would be time-consuming, and I focus on the most commonly enriched GO:BP terms. Interestingly, 7 of the 10 proteins in the most frequent genes in cited studies qualify as H-NBs; these proteins are *BIN1*, *CLU*, *CD33*, *MS4A6A*, *PTK2B*, *EPHA1*, and *SORL1*. The $\bar{x}$ + 2 SD cutoff was used to identify the strictest N-HBs, leaving three proteins to be recognized as H-NBs: *CLU* (k = 45, *BC* = 1541.986), *BIN1* (k = 40, *BC* = 1012.724), and *PSEN1* (k = 39, *BC* = 1452.863). Earlier research supports the notion that proteins considered to be H-NBs (or simply "hubs") follow the "centrality-lethality" rule proposed by Jeong et al. (2001) which states that proteins with high levels of connectivity (i.e. high degrees) are the most essential within a PPI network, and their removal is highly likely to be lethal. Originally established using the yeast *Saccharomyces cerevisiae*, it has been confirmed to be applicable to other eukaryotic organisms, including *Drosophila* (Hahn & Kern, 2004).

Human Protein Network Centrality Analysis – Discussion

Human Hub-Bottlenecks (HBs)

The study of proteins kinases is a substantial topic outside the scope of this thesis. Briefly, proteins kinases are enzymes that selectively modify other proteins via covalent phosphorylation (Adams, 2001; Wang & Cole, 2014). They are involved in a large number of signalling pathways vital to normal physiological functions (Cormier & Woodgett, 2016). Aberrances in individual or multiple protein kinases are associated with a considerable number of diseases, particularly cancer (Brognard & Hunter, 2011; Bhuller et al., 2018) but also cardiovascular disease (Antos et al., 2001; Dorn & Force, 2005) and kidney disease (Li & Gobe, 2006; Rajani, Pastor-Soler, & Hallows, 2017). Tau hyperphosphorylation is another such consequence of dysfunctional kinases.

There is evidence that the eight proteins influence tau phosphorylation to varying degrees, either directly or indirectly. *BDNF* indirectly regulates tau phosphorylation via the PI3K/AKT signalling pathway by activating tropomyosin receptor kinase B (*TRKB*; Elliot, Atlas, Lange, & Ginzburg, 2005). Levels of *BDNF* are decreased in post-mortem AD patients and correlate with higher levels of phosphorylated Tau (Bharani et al., 2020); increasing *BDNF* expression attenuates the effects of tau-related pathology (Jiao et al., 2016). The link between tau phosphorylation and *EGFR* is less certain, but it is plausible that *EGFR* indirectly modifies tau via downstream activation of various pathways. The PI3K/AKT and MAPK pathways are particularly notable (Arcaro et al., 2000; Garay et al., 2015; Shi et al., 2016), as both have been cited in the hyperphosphorylation of tau (Sheng et al., 2001; Sawamura et al., 2003; Wang et al., 2015).

ACTB is arguably an "odd one out" of the eight proteins, as there is no clear evidence indicating it has a role in tau phosphorylation. However, it has been demonstrated to be involved in AD pathology. *ACTB* encodes for beta-actin, a protein that alongside other actins forms microfilaments known as F-actin. Tau binds directly to F-actin (He et al., 2009; Elie et al., 2015), and synergistic dysfunction in F-actin and phosphorylated tau result in tau-based

neurodegeneration (Fulga et al., 2007; DuBoff, Götz, & Feany, 2012; Bardai et al., 2018). Furthermore, actin seems to also be regulated by protein kinase phosphorylation (Prat, Bertorello, Ausiello, & Cantiello, 1998; Papakonstanti & Stournaras, 2002). The *DLG4* protein (referred to as *PSD-95* in the literature) is similar: it lacks evidence suggesting a part in tau phosphorylation, but occurs alongside pathogenic tau (Leuba et al., 2008; Shao et al., 2011) and its function is modulated by kinase-induced phosphorylation (Morabito, Sheng, & Tsai, 2004; Nelson et al., 2013).

The *SRC* protein is the most influential protein here, as it affects tau and some of the HBs and other proteins within the network. It physically interacts with tau (Newman, Gard, Band, & Panchamoorthy, 1998; Reynolds et al., 2008) and possibly phosphorylates it (Derkinderen et al., 2005), while the closely related protein, *FYN*, phosphorylates tau and has been implicated in AD pathology (Lee et al., 2004; Li & Götz, 2017). *SRC* also phosphorylates *EGFR* (Stover, Becker, Liebetanz, & Lydon, 1995; Sato K.I., Sato A., Aoto, & Fukami, 1995; Biscardi et al., 1999), affecting downstream pathways such as STATs which are involved in cell proliferation and cell death (Xi et al., 2003; Sato et al., 2003).

The relationship between *BDNF* and *SRC* is inverse compared to the previously described interactions, in that the former indirectly modulates the latter via *TRKB* activation (Huang & McNamara, 2010). This *BDNF-TRKB-SRC* pathway has been linked to a number of neurological processes, including cortical glutamate release (Zhang et al., 2012) and prevention of astrocytic cell death (Saba et al., 2018). Perhaps more interestingly, *BDNF* regulates axonal guidance and neurite outgrowth through this pathway (Yao et al., 2006; Gavaldà, Gutierrez, & Davies, 2009); specifically, *BDNF* signals *SRC*-dependent phosphorylation of zipcode binding protein 1 (*ZBP1*; Sasaki et al., 2010), which regulates beta-actin translation in dendrites and thus controls dendritic development (Perycz et al,

2011). It has been established that tau pathology occurs in neurites, particularly dendrites (Merino-Serrais et al., 2013; Hall et al., 2015; Kandimalla et al., 2018).

Dysfunction in any of the proteins discussed above could potentially affect tau *in vivo*. However, given its centrality and previously described interactions with both tau and actin, it could be argued that *SRC* is the most important protein in this pathway. It could be hypothesised that aberrances in *SRC* lead to tau-induced dendritic abnormalities, either by abnormally phosphorylating other proteins involved in cytoskeletal dynamics, altering the signalling pathway, or even a combination of both. Several other proteins involved in protein kinase related processes are also present in the network, including *DUSP1, CAMK4*, and *PRKCB*. Given this and the literature, it could be hypothesised that protein kinases are the primary signalling mechanisms by which protein complexes communicate with one another in this AD network, regulated by these eight proteins to varying degrees. Perturbations in these proteins would consequently have an adverse effect on the larger network, leading to disruptions in other processes.

Human Nonhub-Bottlenecks (NH-Bs)

Of the six proteins listed, *HNRNPK* and *PTBP1* are the most relevant to RNA processes. Both *PBTP1* and *HNRNPK* interact physically (Kim et al., 2000), and form complexes along with other heterogeneous nuclear ribonucleoproteins (hnRNPs) that primarily process pre-mRNA through transcription, translation, export, and localisation (Dreyfuss, Kim, & Kataoka, 2002). While both bind to pre-RNA, they appear to process it differently. *PTBP1* is a splicing factor prominently found in alternative splicing, acting as a regulator that binds to intronic polypyrimidine tracts adjacent to target exons (Patton, Mayer, Tempst, & Nadal-Ginard, 1991; Lin & Tarn, 2005), while *HNRNPK* is multifunctional and implicated in most

major pre-RNA processes (Bomsztyk, Denisenko, & Ostrowski, 2004), including transcription activation (E.F. Michelotti, G.A. Michelotti, Aronsohn, & Levens, 1996).

PTBP1 regulates normal tau splicing, and acts as a strong inclusion inhibitor for exons 2, 6, and 10, (Wei, Memmott, Screaton, & Andreadis, 2000; Li, Arikan, and Andreadis, 2003) while its effects on exon 3 are dependent on the latter's splicing status (Arikan et al., 2002). More importantly, it has been linked directly to the formation of tau pathology by missplicing exon 10, resulting in the inherited tauopathy frontotemporal dementia (Wang et al., 2004). Yasojima et al.(1999) found that tau mRNAs containing exon 10 were heavily upregulated in AD brains with heavy burdens of NFTs, compared to mRNAs lacking exon 10. The relationship between *HNRNPK* and tau is less clear, but a study by Liu and Szaro (2011) suggests it co-regulates tau and other cytoskeletal proteins post-transcriptionally; knockdown of *HNRNPK* led to severe deficits in axon outgrowth and cytoskeleton organization. Furthermore, Hutchins and Szaro (2013) demonstrated that *HNRNPK* is phosphorylated by c-Jun N-terminal kinase (*JNK*) and deficits in both genes led to the same dysfunctions described in Liu and Szaro's study. Interestingly, *HNRNPK* is also phosphorylated by cyclin-dependent kinase 2 (*CDK2*), which also phosphorylates tau (Moujalled et al., 2015).

Other hnRNPs have been shown to be both involved in tau exon 10 splicing (Hartmann et al., 2001; Wang et al., 2010; Liu et al., 2020) and localised to NFTs in post-mortem AD brains (Ishikawa et al., 2004; Mizukami et al., 2005). Given this, any potential pathogenicity of the *PBTP1* and *HNRNPK* proteins in tau-specific pathology should be studied with other hnRNPs and splicing factors, as abnormal RNA processing is more likely to occur in a complex rather than from a singular product.

The *EEF1A1* protein's is primarily involved in protein translation, namely by mediating the transporting aminoacyl-tRNA to the A site of the ribosome during protein synthesis (Browne

& Proud; 2002; Mateyak & Kinzy, 2010). It is also involved in cytoskeletal organization, bundling, binding, and regulating actin filaments (Murray et al., 1996; Gross & Kinzy, 2005; Bunai et al., 2006; Gillardon, 2009). However, research supporting a connection to tau is scarce: Malmqvist, Anthony, and Gallo (2013) found tau mRNA containing axonal granules is associated with *EEF1A1*, while Meier et al., (2016) detected an association between the two proteins in both normal and AD brains. The latter result is interesting because *EEF1A1* has been mostly associated with Aβ pathology in AD studies (Beckelman et al., 2016; Beckelman et al., 2016). Further study is required to elucidate whether *EEF1A1* interacts with tau. Given its effects on actin and other cytoskeleton proteins, dysfunctional *EEF1A1* could influence tau indirectly via abnormal cytoskeletal organisation.

Given the three proteins above serve similar functions, *SOD2* and *BECN1* could be viewed as outlying NH-Bs. *SOD2* encodes an enzyme that catalyses the transformation of superoxide ($O_2^{\bullet-}$), a byproduct of oxidative phosphorylation, to hydrogen peroxide (H_2O_2), preventing cellular damage caused by oxidative stress (Murphy, 2009). Oxidative stress has long been proposed as a causative factor of AD pathology (Smith et al., 2000; Wang et al., 2014), and research suggests dysfunctional *SOD2* expression contributes to this. Both Massaad et al. (2010) and Melov et al. (2007) found that reduced *SOD2* expression led to increased amyloid burden and hyperphosphorylated tau. Massaad et al. (2009) also showed that *SOD2* overexpression ameliorated AD-related pathology, as well as memory and learning deficits. How exactly *SOD2* dysfunction affects tau phosphorylation, and by tau pathology, requires further research.

BECN1 is central to autophagy, regulating it as part of phosphatidylinositol 3-kinase (PI3K-III) complexes (Itakura et al., 2008; Matsunaga et al., 2009). It appears to have a role in AD, but it is more involved with amyloid-β than tau (Pickford et al., 2008; Lucin et al., 2013; Swaminathan et al., 2015). Nevertheless, a few studies have shown that *BECN1* expression is

significantly decreased in tauopathies and in cells overexpressing pathogenic tau (Ando et al., 2016; Mahendran et al., 2020). It is also important in neuronal development and differentiation acting as a repressor of transcription factors such as *Pbx1* and its paralog *PTBP2* in embryonic stem cells (Linares et al., 2015) and in neuronal differentiation, where *PTBP1* expression is reduced by the microRNA miR-124 (Makeyev et al., 2007), increasing *PTBP2* expression and initiating neuronal maturation (Licatalosi et al., 2012; Li et al., 2014; Vuong et al., 2016).

Human Hub-Nonbottlenecks (H-NBs)

The precise basis of the rule has been debated (He & Zhang, 2006; Zotenko et al., 2008), but this argument is outside the remit of this thesis. Chandramohan et al., (2021, preprint) also found this rule applies to the human interactome, finding 73% of H-NBs (referred to as "pure hubs" (PHs)) to be essential, compared to 23% of HBs/MX and 47% of NH-Bs/PBs. In their study, N-HBs/PHs are enriched in biological processes relating to nucleic acid and protein metabolism, transcription, and DNA repair. This is not the case with the 24 H-NBs here, with terms relating to immunity being the most significantly enriched. Such terms include "positive regulation of immune system process", "regulation of immune response", "leukocyte activation", "immune effector process" (all $n = 11$, $p = 2.66\times10^{-6}$), and "regulation of immune response" ($n = 13$, $p = 2.66\times10^{-6}$). The link between AD and the immune system was examined in the GO enrichment analysis of the whole human network but since immunity was not highlighted in the *Drosophila* network. It is not worthwhile in this thesis to focus on a biological process not relevant to *Drosophila*.

Again, no immune-related terms were enriched amongst the three identified $\bar{x}$ + 2 SD H-NBs, with the following terms found to be significantly enriched instead: "positive regulation of amyloid fibril formation" ($n = 2$, $p = 0.00021$); "negative regulation of amyloid-beta

formation" (n = 2, p = 0.00036); and "positive regulation of supramolecular fiber organization" (n = 3, p = 0.00036). It is important to note that Chandramohan and colleagues (2021, preprint) conducted their analysis using identified NH-Bs/PHs from the whole human genome, so it is more than reasonable to assume that the discrepancy here is due to sample size.

Of the strict H-NBs, *BIN1* is the most interesting. *PSEN1* has been extensively studied in AD, while *CLU*, though there is evidence suggesting it contributes to AD pathology, has no discernible *Drosophila* ortholog. The role of *BIN1* in tau pathogenicity has been discussed previously and is involved in tau-specific pathology, (Chapuis et al., 2013; Lasorsa et al., 2018), and while the specific interaction between its primary endocytic function and tau is unclear, it may promote tau pathology by increasing endocytic flux and internalisation of tau aggregates, (Calafate et al., 2016), before releasing tau via extracellular vesicles (Crotti et al., 2019). The loss of *BIN1* also results in synaptic tau accumulation, thus leading to dendritic abnormalities (Glennon et al, 2020).

Drosophila Protein-Protein Interaction Network

The full *Drosophila* network graphic can be found in Appendix F. 288 genes were entered in STRING, and 260 were recognised in the database. Deleting unconnected proteins results in a total of 224 nodes and 1080 edges. Table 7 displays the average values of network-wide measures.

Table 7. Average Drosophila network-wide statistics.

Network Measure	Average Value
Average No. of Neighbours	9.643

Network Diameter	7
Network Radius	4
Characteristic Path Length	2.919
Clustering Coefficient	0.339
Network Density	0.043
Network Heterogeneity	0.953
Network Centralization	0.196

Small World Property

The small-world property for the *Drosophila* AD network was calculated using the same equation for the AD human network, as proposed by Humphries, Gurney, and Prescott (2006). The results show that $\gamma^i = 8.7$ and $S^i > 7.8$; thus, the *Drosophila* AD network meets the criteria for the small-world law.

Global Network Properties of *Drosophila* Protein-Protein Interaction Network

Like in the human PPIN, the most important global network properties are probably *average no. of neighbours*, *CPL*, and *clustering coefficient*.

As Table 8 shows, the *average no. of neighbours* for the *Drosophila* network is 9.643. While smaller than the human network, it indicates a high level of connectivity between *Drosophila* proteins. The *clustering coefficient* is 0.339, suggesting that proteins in the network have a tendency to form modules. The significance of module in both networks will be discussed in the next analysis. The *CPL* is 2.919 which, according to Xu et al., (2011), is lower than the observed average for *Drosophila*.

Comparison of AD *Drosophila* Network to Random Networks

Like the human AD network, the constructed *Drosophila* network should be significantly different from random. To confirm this, the method employed for the human AD network was reused. 1000 Erdős–Rényi model networks were generated and subjected to the following analyses: degree (K) and betweenness (BC). From these analyses, 224 values (equal to the number of values from the *Drosophila* AD network) were randomly sampled and compared to the *Drosophila* AD network using the Wilcoxon signed-rank test. The full code can be found in Appendix **G.**

Significant differences were found between the *Drosophila* AD network centralities and the random network centralities. Both AD degree and betweenness centralities were significantly different ($p < 0.01$ and $p < 00.4$, respectively) from random, showing that the AD *Drosophila* network is distinct from random.

***Drosophila* AD Network Centrality Analyses**

Table 9. The fifteen proteins with consistently high values across all measures. Bold denotes proteins that are within the top five for two or more measures.

Protein	Degree (*k*)	Closeness	Betweenness	Eigenvector
CaMKI	**54**	**0.002257336**	**6060.100345**	**0.24534941**
Egfr	**51**	**0.002192982**	**4354.052268**	**0.24477025**
Src64B	**46**	**0.002164502**	**3341.023768**	**0.234146833**
dlg1	**44**	**0.002155172**	**3377.189903**	**0.227650864**
Src42A	**41**	**0.002132196**	**1786.157172**	**0.227828207**
Rab5	40	0.002070393	2729.43903	0.2089885
Appl	38	0.002114165	2918.660182	0.204685737
Cam	33	0.002132196	2091.350067	0.19039358
His2Av	31	0.002087683	4683.826798	0.135075231

Syb	29	0.001953125	880.2149527	0.159107441
Psn	24	0.00203252	2183.149952	0.112835954
Sgg	23	0.001945525	933.0215847	0.108647793
Mys	23	0.001824818	609.6096298	0.11192283
Gapdh1	22	0.001937984	2011.260184	0.087323844
F1	22	0.001972387	1264.446853	0.088242871

Table 9 shows that *CaMKI*, *Egfr*, *Src64B*, *dlg1*, and *Src42A* score highly across each measure. *Egfr* and *Src64B* are orthologs of the human genes *EGFR* and *SRC*, respectively, suggesting the importance of these gene products is conserved between the two networks.

As in the human network, to identify proteins from the categories described by Yu et al. (2007), the averages of the centralities plus one or two standard deviations of the mean were used as cutoff values. These values are shown in Table 10.

Table 10. Cutoff values for identifying proteins in each category. Degree values are rounded to their nearest whole number.

	Degree	Betweenness
Mean ($\bar{x}$)	10	427.4667
$\bar{x}$ + 1 SD	19	1229.4413
$\bar{x}$ + 2 SDs	28	2405.9238

Drosophila **Hub-Bottlenecks (HBs)**

Using the $\bar{x}$ + 2 SDs cutoff values, *CaMKI*, *His2Av*, *Egfr*, *dlg1*, *Src64B*, *Appl*, and *Rab5* can be categorised as HBs. According to Chandramohan et al., (2021, preprint) HBs (referred to as "mix proteins" (MX)) are more evolutionary conserved than the other protein categories. All the genes identified as HBs have human orthologs, four of which are also HBs in the human network (*Egfr*/*EGFR*, *Src64B*/*SRC*, *Appl*/*APP*, and *dlg1*/*DLG4*). In regards to GO

enrichment, Chandramohan et al. (2021, preprint) focused only on human genes, so terms that are enriched among human HBs may not be enriched in these *Drosophila* proteins.

This appears to be the case, as GO:BP terms relating to development are the most enriched in these proteins, and include "dorsal closure" ($n = 4$, $p = 9.63\times10^{-5}$), "establishment or maintenance of apical/basal cell polarity" ($n = 3$, $p = 0.00025$), "morphogenesis of follicular epithelium" ($n = 3$, $p = 0.00025$), "regulation of cell population proliferation" ($n = 4$, $p = 0.00025$), and "ovarian follicle cell development" ($n = 4$, $p = 0.00025$).

Drosophila Nonhub-Bottlenecks (NH-Bs)

Using the $\bar{x} + 1$ cutoff, four proteins can be categorised as NH-Bs in *Drosophila*: *Sod2* (k = 19, BC = 1733.243), *CG17528* (k = 9, BC = 1352.871), *Fs(2)Ket* (k = 19, BC = 1349.684), and *Vap33* (k = 14, BC = 1266.533). Only *Sod2* has a human ortholog that is also an NH-B. A GO enrichment analysis could not be performed for these four proteins likely due to the lack of recorded or predicted interactions amongst them.

Drosophila Hub-Nonbottlenecks (H-NBs)

Employing the $\bar{x} + 1$ cutoff resulted in 18 proteins being identified as H-NBs. Again, examining each protein individually would be laborious, therefore they will be discussed within the context of their most significant GO:BP terms. Four proteins are H-NBs in both species' networks (*mys*/*ITGB1*, *Eph*/*EPHA1*, *cathD*/*CTSD*, and *Amph*/*BIN1*). Using the $\bar{x} + 2$ cutoff, only one protein could be categorised as a strict H-NB, namely *Syb*. Unlike the human H-HBs, most enriched GO:BP terms among the *Drosophila* H-NBs relate to development. However, terms relating to vesicle-based processes are also significantly enriched, including but not limited to "exocytosis" ($n = 7$, $p = 2.12\times10^{-8}$), "vesicle-mediated transport" ($n = 8$, p

= 4.78×10^{-6}), and "establishment of vesicle localization" (n = 5, p = 6.12×10^{-6}). As shown by a previous analysis, both species' whole networks had at least one term relating to vesicle-associated processes significantly enriched. Combined with the presence of a conserved protein module primarily enriched in endocytic processes (discussed in the next section), it is more than appropriate to hypothesise endocytosis as a conserved mechanism in AD pathology.

Drosophila Network Centrality Analysis – Discussion

Drosophila Hub-Bottlenecks (HBs)

The question is whether these proteins have any interactions with *Drosophila* tau (dTau) *in vivo*, or if they at least share functions in processes that involve protein kinases. Evidence for the former, outside of the genetic screens cited for this project, is lacking. However, there are studies that support phosphorylation-related functions in a few of these proteins. For example, like human *EGFR*, *Drosophila Egfr* activates several kinase cascades that activates pathways such as MAPK (Cela & Llimargas, 2006; Moses, C. et al., 2011; Jiang et al., 2011), which phosphorylates a range of substrates (Peverali et al., 1996; Nir et al., 2012; Auer et al., 2015). As previously stated, the MAPK pathway has been cited in the normal and abnormal phosphorylation of tau in humans; however, evidence of this occurring in *Drosophila* is lacking.

CamkI, the ortholog of *CAMK1D*, is unusual in that most of its functions have been inferred via phylogenetic similarity and not experimentally confirmed, according to FlyBase. Its paralog *CamkII* has been shown to phosphorylate proteins (Wang et al., 2002; Yang et al., 2010) and itself. Additionally, it is active in several neuronal processes, including synaptic transmission (Haghigi et al., 2003) and plasticity (Andersen et al., 2005). Why *CamkI* was

identified as a HB is unknown. *Dlg1* appears to share the same neuronal functions as its human ortholog *DLG4* (Karunanithi et al., 2002; Kumar et al., 2009) and is also regulated by kinase phosphorylation, including by *CamkII* (Koh et al., 1999; Zhang et al., 2007). *Rab7* forms part of a larger group this study has found to be conserved between both networks and will be discussed in the next section.

Like its human counterpart, *Src64B* is the most central protein within its network. It regulates several pathways via protein phosphorylation, including MAPK (Xia et al., 2008), JNK, STAT, and PI3K (Read et al., 2004; Poon et al., 2018). Studies in other organisms strongly implicate these pathways in tau-specific pathology, either directly or indirectly (Atzori et al., 2001; Sawamura et al, 2001; Baki et al, 2004; Yoshida et al., 2004; Vogel et al., 2009; Colodner & Feany, 2010; Ploia et al., 2011; Cai et al., 2011; Wang et al., 2015; Maphis et al., 2016). Whether *Src54B* has a role in the pathogenesis and/or propagation of tau pathology in *Drosophila* through the modulation of these same pathways has not been established.

There is a little evidence of the relationship between *Src64B* and tau, with only Feuillette et al. (2020) finding that loss-of-function of *Src64B* and its paralog *Src42A* enhances tau toxicity. Like its human counterpart *Src64B* modulates actin dynamics, primarily in ring canal morphogenesis (Dodson et al., 1998; Kelso et al., 2002). Furthermore, as previously mentioned *SRC* is involved in axonal guidance, also a function of *Src64B* (Nicolai et al., 2003). However, both these processes are activated by *BDNF* signalling which has no recorded ortholog in *Drosophila*, so it remains unknown whether *Src64B* acts independently in these processes, or it is regulated by either another protein or as-yet unidentified species-specific ortholog of *BDNF*.

Drosophila Nonhub-Bottlenecks (N-HBs)

Sod2 is a conserved NH-B in both human and *Drosophila* networks, and its function is virtually the same in both species. It appears *Sod2* expression has considerable biological consequences in *Drosophila*, with knockout resulting in early mortality in young adult flies (Kirby et al., 2002; Duttaroy et al., 2003). Downregulation of *Sod2* also has detrimental effects in the fly nervous system, causing neuronal dysfunctions including neurodegeneration (Paul et al., 2007; Celotto et al., 2012; Oka et al., 2015). *Sod2* has been linked directly to tau pathology as part of a wider, mediating group of proteins involved in oxidative stress (Dias-Santagata et al., 2007).

Fs(2)Ket is the ortholog of human *importin-β*, encoded by the gene *KPNB1*. Both proteins share similar functions, primarily acting as nuclear transport receptors (Lippai et al., 2000; He et al, 2017). The relationship between aberrant nuclear transport and tau has been reviewed by Diez and Wegmann (2020), but there is little evidence supporting any interactions between either *Fs(2)Ket* or *importin-β* and tau; however, Nuovo et al. (2018) did observe that the latter co-localised with hyperphosphorylated tau in post-mortem brains. *Vap-33* and its human ortholog are both present in a conserved protein module, and will be discussed in the next section. According to FlyBase, *CG17528*'s closest ortholog is *DLCK1/DLCK2*, though for this study it was assumed to be the counterpart of *CAMK4*. Its functions have been inferred from structural or sequence similarity, and is predicted to be involved in processes relating to calmodulin binding and protein phosphorylation.

Drosophila Hub-Nonbottlenecks (H-NBs)

The product of *mys, βPS*, functions similar to its human ortholog, acting as a receptor for the extracellular protein laminin and regulating cellular adhesion (Gotwals et al., 1994; Zhang et al., 2010; Egoz-Matia et al., 2011). It also interacts with actin, affecting its assembly and

overall dynamics (D. Fristrom et al., 1993; Bateman et al., 2001; Delon & Brown, 2009). *Eph* protein is a receptor protein tyrosine kinase (PTK) with a number of roles in the *Drosophila* nervous system, particularly during axons guidance (Scully et al., 1999; Dearborn et al., 2002; Boyle et al., 2006). Though direct evidence of its functions in pathological states is scant, *cathD* appears to be primarily involved in apoptosis via lysosome proteolysis (Kinser & Dolph, 2012; Zhang et al., 2020).

Amph is the most confounding protein. Despite being strongly homologous with *BIN1*, *Amph* lacks its endocytic functions, the central domain in exon 8 which contains motifs that bind proteins crucial to endocytosis, such as clathrin and AP2 (Owen et al., 1998; Ramjaun & McPherson, 2002), is poorly conversed between *BIN1* and *Amph* (Leventis et al., 2001). *Amph* proteins are absent from presynaptic terminals (Zelhof et al., 2001), where endocytosis primarily takes place (Royle & Lagnado, 2010), and synaptic transmission in *Drosophila* with Amph mutations is largely unaffected (Razzaq et al., 2001). In contrast, *Drosophila* expressing mutated endocytic proteins, such as *Vps35*, have noticeable defects in signalling and protein localisation (Korolchuk et al., 2007), as well as increased lethality (Olswang-Kutz et al., 2009).

Excluding *Amph*, *cathD* is the only one that directly interacts with tau in a pathological state. Khurana et al. (2010) found that upregulation of *cathD* significantly increased tau neurotoxicity in *Drosophila*. Though not concerning tau, Myllykangas et al. (2005) showed *cathD* mutations led to neurodegeneration in *Drosophila*, suggesting that *cathD* dysfunction is detrimental to the brain. Evidence of a relationship between tau and *mys* or *Eph* is virtually non-existent. Given the former interacts directly with the cytoskeleton and the latter influences neuronal development, it would be prudent to focus on these proteins and their possible roles in *Drosophila* models of AD.

Amph mediates tau toxicity in *Drosophila* (Chapuis et al., 2013). How this works is unclear, but Dräger et al. (2017) provided a potential mechanism, there is an association between tau neurodegeneration and F-actin accumulation. Dräger et al., found that *BIN1* and *Amph* (*dBin1* in the paper) bind actin filaments via their BAR domains, stabilizing them affecting their bundling. More importantly, downregulation of *Amph/dBin1* led to decreased levels of actin-rich rods, particularly those induced by tau. The BAR domain is well-conserved between *BIN1* and *Amph*, (Casal et al., 2006) as is their ability to regulate the formation and maintenance of muscle transverse tubules (Razzaq et al., 2001; Fugier et al., 2011; Safi et al., 2016; Fu & Hong, 2016). It is possible that these proteins directly interact with elements of the cytoskeleton, and when dysfunctional either cause or contribute to pathophysiology relating to tau. This requires more research.

Conserved Modules Between Human & *Drosophila* PPINs

My analysis has identified the most important individual proteins in both the human and *Drosophila* networks, as well as their strongest interactions and likeliest functions. Furthermore, it has underlined conserved pathways between the species' networks. However, proteins rarely exert their effects without influencing or being influenced by other proteins. If one were to grossly simplify them, then it could be biological processes are sequential protein interactions that result in either local or global physiological outcomes; this could also be argued for diseases. These conserved modules or complexes represent potentially conserved processes or cellular machinery, respectively that could be considered more widely as potential avenues for developing strategies for understanding AD. These networks were selected based on their size, consistency, and accuracy across multiple executions of the GASOLINE software. Based on my analysis, I have identified 4 such networks that could be

pivotal to the progression of neurodegenerative diseases and provide a focus for future strategies for research into new therapeutic targets and novel disease mechanisms

Conserved Module 1 – "Vesicle Processing Group"

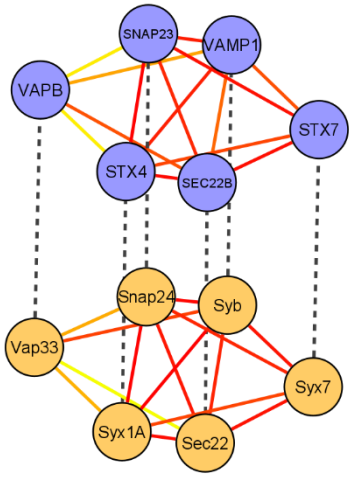


Figure 8. GASOLINE-generated network of conserved module 1.

The **Vesicle Processing Group** consists of the proteins SEC22B, SNAP23, STX4, STX7, VAMP1, and VAPB (purple) in the human network and the proteins Sec22, Snap24, Syx1A, Syx7, Syb, and Vap33 (yellow) in the *Drosophila* network. These modules are named due to the significant enrichment of GO:BP terms concerning vesicle-related processes, as illustrated by Figures 9 & 10

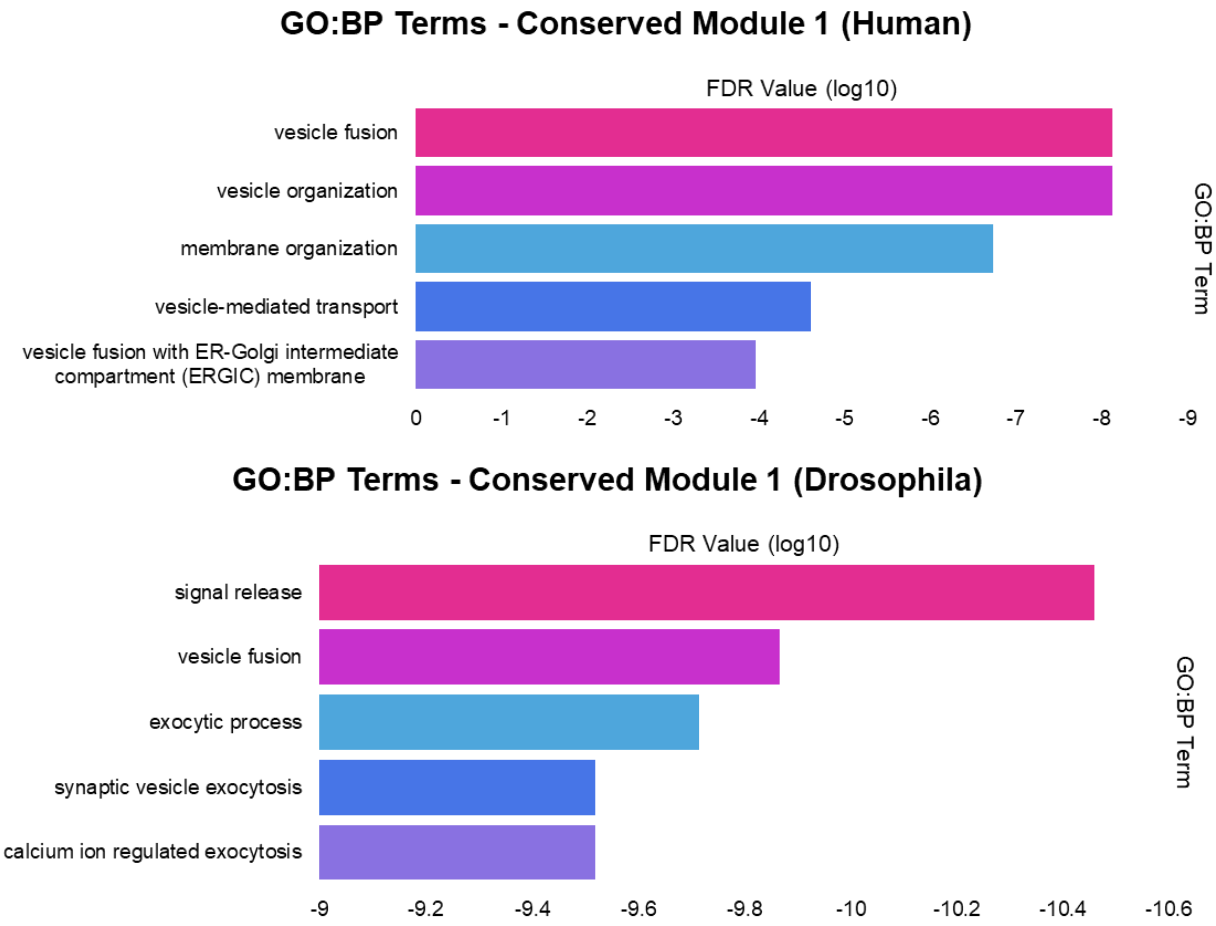


Figure 9 & Figure 10. Bar graphs displaying the most significantly enriched GO:BP terms in both humans and Drosophila for CM1.

Conserved Modules 2 – "Splicing Assemblies"

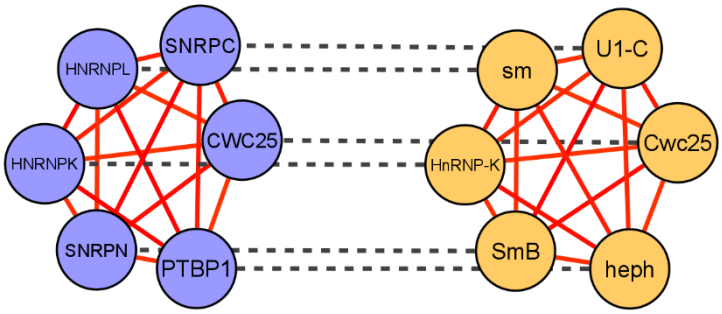
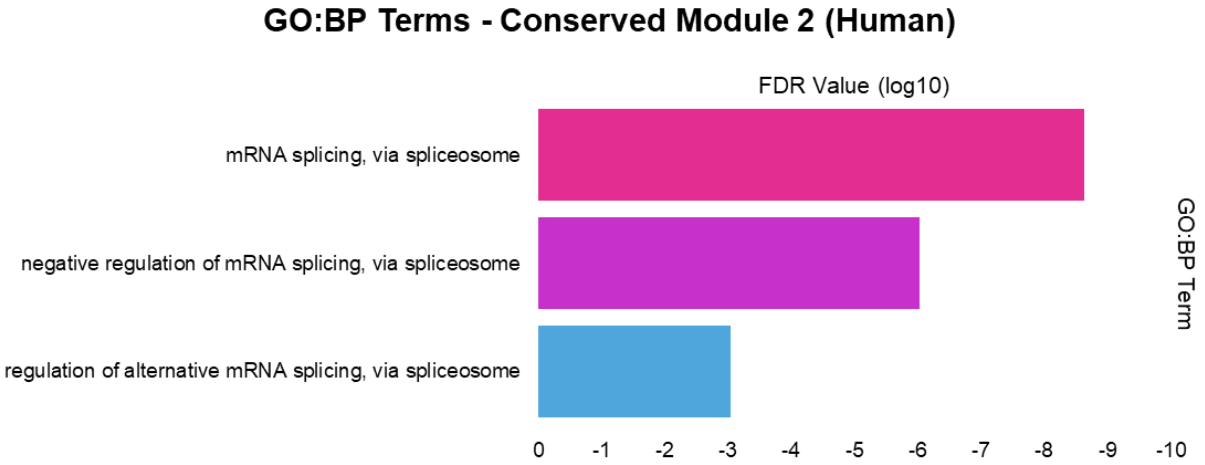


Figure 11. GASOLINE-generated network of conserved module 2.

The **Splicing Assemblies** Modules consists of the proteins CWC25, HNRNPK, HNRNPL, PTBP1, SNRPC, and SNRPN in the human network (purple) and the proteins Cwc25, HnRNP-K, sm, heph, U1-C (shortened from snRNP-U1-C), and SmB in the *Drosophila* network (yellow). These modules are referred to "Splicing Assemblies" due to the enrichment of GO:BP terms relating to mRNA splicing, as depicted by Figures 12 & 13.



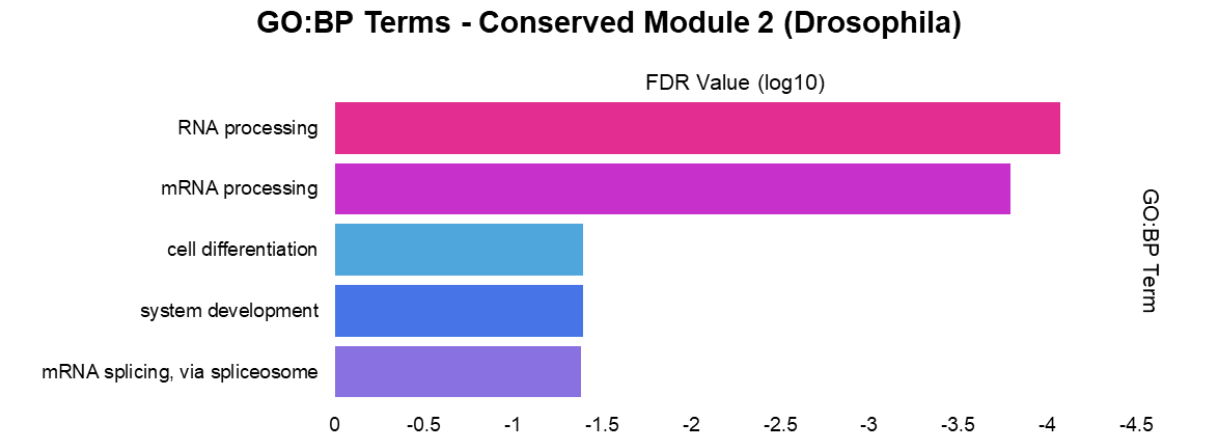


Figure 12 & Figure 13. Bar graphs displaying the most significantly enriched GO:BP terms in both humans and Drosophila for CM2.

Conserved Modules 3 – "RAB Groups"

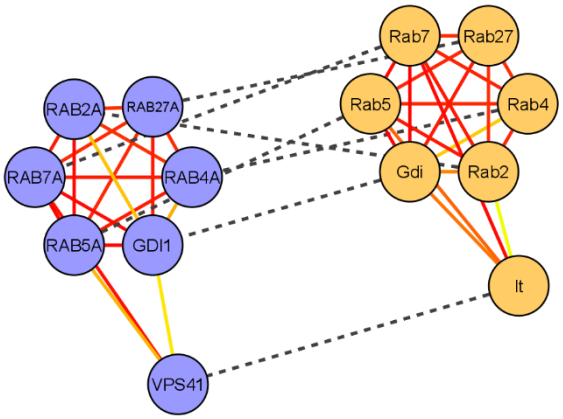
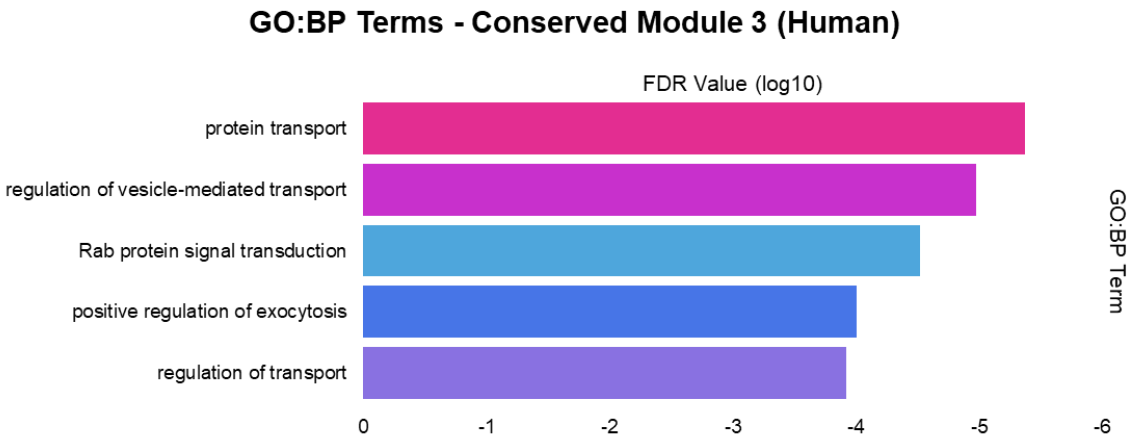


Figure 14. GASOLINE-generated network of conserved module 3.

"Conserved Module 3" consists of the proteins GDI1, RAB2A, RAB4A, RAB5A, RAB7A, RAB27A, and VPS41 in the human network (purple) and the proteins Gdi, Rab2, Rab4, Rab5, Rab7, RAB27, and lt in the *Drosophila* network (yellow). Unlike the previous modules, these modules are named after the RAB protein family, as most proteins in both modules belong to this family. The most significantly enriched GO:BP terms for each module are illustrated in Figures 15 and 16.



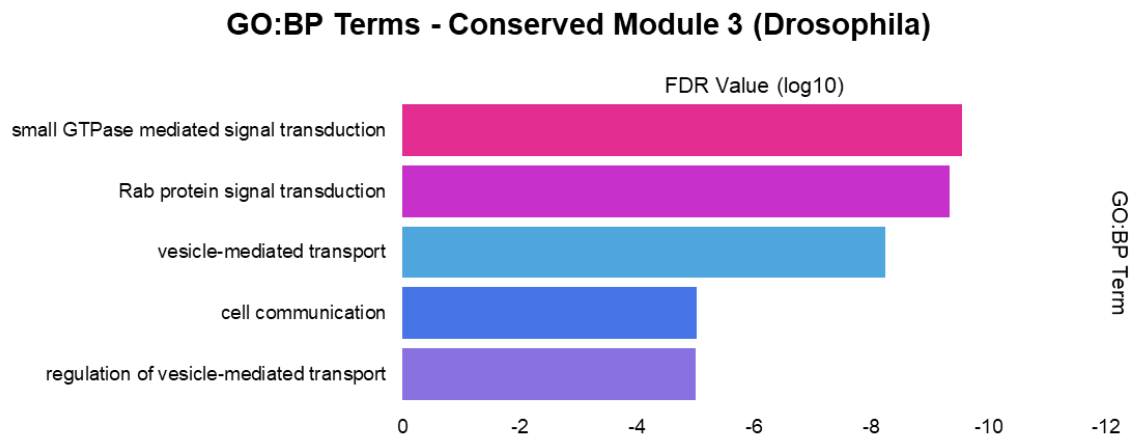


Figure 15 & Figure 16. Bar graphs displaying the most significantly enriched GO:BP terms in both humans and Drosophila for CM3.

Conserved Module 4 – "Ribosomal Protein Complexes"

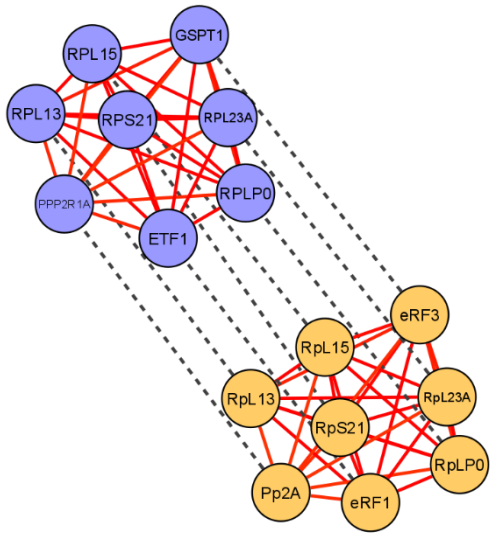


Figure 17. GASOLINE-generated network of conserved module 4.

"Conserved Modules 4" (CM4) are the largest conserved modules, and consist of the proteins ETF1, GSPT1, PPP2R1A, RPLP0, RPL13, RPL15, RPL23A, and RPS21 in the human network (purple) and the proteins eRF1, eRF3, Pp2A, RpLP0, RpL13, RpL15, RpL23A, and RpS21 in the *Drosophila* network (yellow). Like previous modules, these modules are named

after the most prevalent protein family, in this case the ribosomal proteins. These modules are enriched with GO:BP terms relating to protein translation, as shown in Figures 18 and 19.

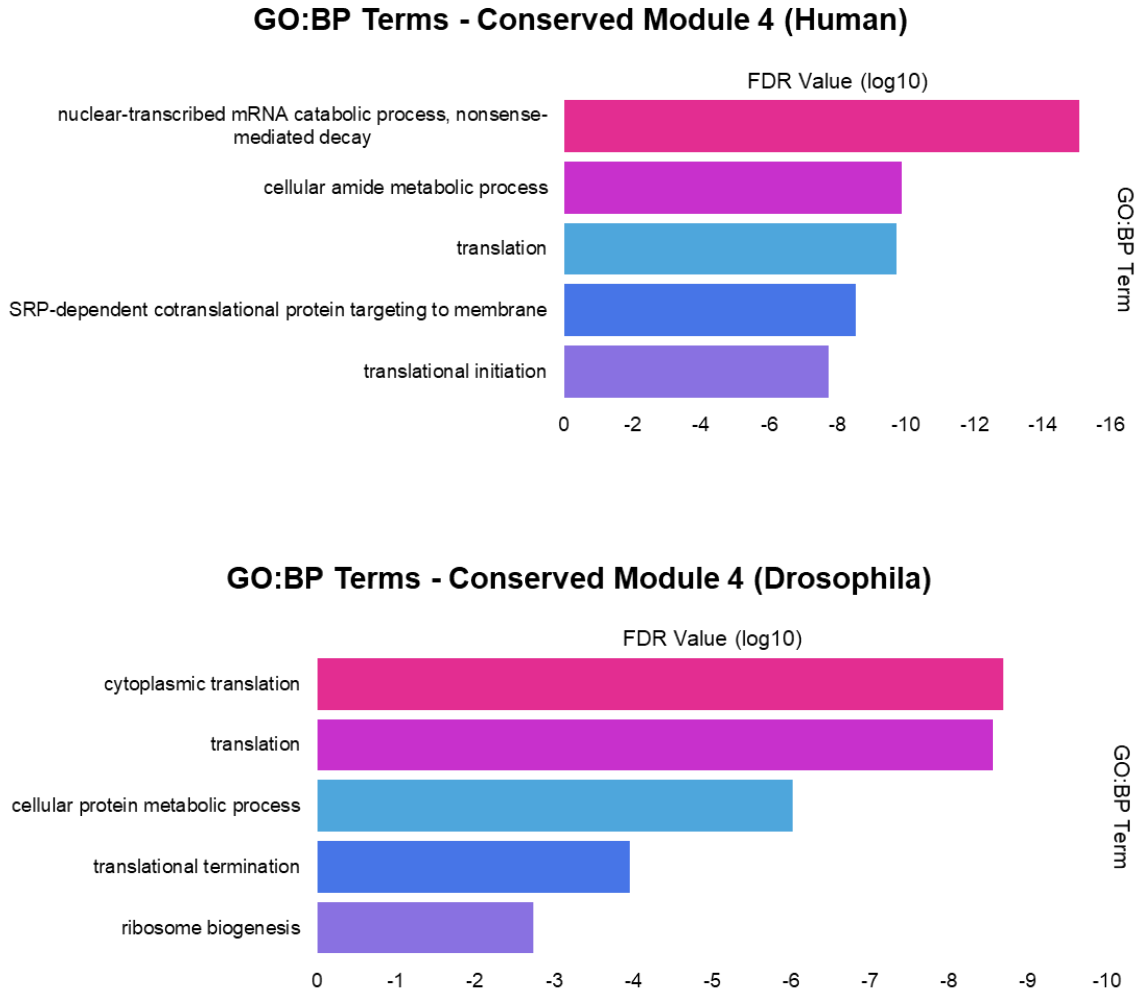


Figure 18 & Figure 19. Bar graphs displaying the most significantly enriched GO:BP terms in both humans and Drosophila for CM4.

Discussion

Vesicle Processing Group

These modules support my previous analysis of GO:BP enrichment in both species' whole networks. The *Drosophila* module also contains two proteins identified in the previous section as potential candidate proteins: *Vap33* (an NH-B) and *Syb* (an H-NB). Most proteins

in both modules belong to the SNARE protein family. SNARE proteins primarily mediate the fusion of vesicles to the membrane and exocytosis (Wang et al., 2017). SNARE proteins are categorised as one of two subclasses: Q-SNAREs and R-SNARES (Fasshauer et al., 1998). Three Q-SNAREs and one R-SNARE form a four-helix bundle, the SNARE complex (Poirier et al., 1998; Chen et al., 2002), that is primarily responsible for fusing the vesicle membrane with the target membrane (Südhof & Rothman, 2009; Van Den Bogaart et al., 2010).

Most of the individual proteins are involved in exocytosis and membrane fusion, namely the syntaxins *STX4* and *STX7,* (Hammarlund et al., 2007; Woodbury & Rognlien, 2013; Nakamura et al., 2000; Mullock et al., 2017), *SNAP23* (Ravichandran et al., 1996; Dong & Whiteheart, 1999; Chieregatti et al., 2004), *VAMP1* (Rao et al., 2004; Ward et al., 2017), and *SEC22B* (Hay et al., 1997; Zhang et al., 2017). Interestingly, these proteins have significant roles in the nervous system relating to their primary functions. *STX4* and *STX7* act in hippocampal neurons, in recycling AMPA receptors and endosomal membrane fusion, respectively (Mohanasundaram & Shanmugam, 2010; Kennedy et al., 2010; Mori et al., 2020).

SNAP23, while not as widely expressed in the brain as closely related *SNAP25* (Chen et al., 1999; Hepp et al., 1999), is enriched in dendritic spines, colocalizing with NMDA receptors. Furthermore, *SNAP23* can substitute for *SNAP25* and perform its functions if the latter is knocked out or not expressed (Delgado-Martínez et al., 2007; Arora et al., 2017). *VAMP1* participates in neurotransmitter release (Trimble et al., 1988; Raptis et al., 2005) and optimises synaptic transmission at neuromuscular junctions (NMJ) (Liu, Sugiura, & Lin, 2011); furthermore, it forms part of a SNARE complex with STX4 and *SNAP25* that regulates the exocytosis of NMDA receptors (Gu & Huganir, 2016). *SEC22B* appears to aid neurite growth by contributing to plasma membrane expansion (Petkovic et al., 2014; Gallo et al., 2020).

VAPB is the only protein here not classed as a SNARE protein, belonging to the *VAP* protein family. *VAP* proteins share multiple functions, including exocytosis of neurotransmitters (Skehel et al., 1995), lipid transportation and metabolism (Kawano et al., 2006; Wakana et al., 2015), and ER-Golgi complex trafficking (Prosser et al., 2008; Peretti et al., 2008). While there is no evidence they are directly involved in vesicle fusion or exocytosis, VAP proteins do interact with SNARE complexes, especially VAMPs.VAPB mediates the unfolded protein response, an ER response to accumulated misfolded proteins (Kanekura, Nishimoto, Aiso, & Matsuoka, 2006; Gkogkas et al., 2008).

The functions of the *Drosophila* proteins have mostly been inferred by phylogenetic similarity to human proteins, a common theme throughout this thesis. Nevertheless, there is experimental evidence from *in vivo* studies on *Drosophila*. *Syx4*, the ortholog of *STX4*, regulates neurotransmitter release (Harris et al., 2018) and synaptic plasticity, the latter by trafficking the proteins synaptotagmin 4 (*Syt4*) and neuroligin (*Nlg1*) (Harris et al., 2016). *Syx7* is also known as *Avalanche* (*avl*), and is required for vesicle fusion, to form early endosomes (Lu & Bilder, 2005; Morrison et al., 2008).

Evidence of the role of *Snap24*, the ortholog of *SNAP23*, is scarce, but Niemeyer, and Schwarz (2000) suggest it retains its exocytic role. It can also substitute for *Snap25* if the latter is not present (Vilinsky et al., 2002). *Sec22* maintains the ER-Golgi trafficking function of its human relative (Zhao et al., 2015; Lakatos et al., 2019). *Syb*'s functions are mostly inferred, but evidence suggests neuronal *Syb* is required for synaptic exocytosis (Bhattacharya et al., 2002; DeMill et al., 2014). *Vap33* has more neuronal functions compared to *VAPB*, affecting synaptic morphology (Forrest et al., 2013), bouton formation (Pennetta et al., 2002), and trafficking to axons (Yang et al., 2012).

Splicing Assemblies

The relevance of these modules stems from their involvement in alternative splicing. As tau protein and its isoforms are formed by this process. Additionally, the human module contains two proteins (*HNRNPK* and *PTBP1*, both NH-Bs) identified as proteins for further study.

Most, if not all, proteins in both modules are RNA-Binding Proteins (RBPs). RBPs bind to RNA via structural motifs such as RNA recognition motif (RRM), hnRNP K homology domain (KH), and zinc finger (ZF), and the vast majority are involved in protein synthesis, whether directly or indirectly (Lunde et al., 2007; Gerstberger et al., 2014). The interaction of RBPs with RNA typically forms dynamic complexes known as ribonucleoprotein particles (RNPs) (Mitchell & Parker, 2014). Most proteins in both modules form such complexes and can be divided into two categories: small nuclear ribonucleoproteins (snRNPs) and heterogeneous nuclear ribonucleoproteins (hnRNPs). *SNRPC*/*snRNP-U1-C* and *SNRPN*/*SmB* fall into the former category, while *HNRNPK*/*HnRNP-K*, *HNRNPL*/*sm*, and *PTBP1*/*heph* are classified as the latter. The status of *CWC25* is uncertain.

snRNPs are complexes consisting of proteins (such as *SNPRC*) and small nuclear RNAs (Kiss, 2004). The snRNPs combine with one another to form the spliceosome, a large RNP complex that catalyses pre-mRNA splicing (Matera & Wang, 2014). *SNRPC* encodes for a component of the U1 snRNP, also known as the U1-specific protein C, which selectively binds to the 5' of pre-mRNA and initiates spliceosome assembly (Mount et al., 1983; Ruby & Abelson, 1988; Seraphin & Rosbash, 1989). U1-specific protein C is crucial in initial pre-mRNA 5' splice-site recognition and binding (Heinrichs et al., 1990; Rossi et al., 1996). It also seems to stimulate early splicing complex formation (Will et al., 1996). The exact function of *SNRPN* is less clear, but it does seem to be involved in pre-mRNA splicing (McAllister, Amara, & Lerner, 1988). However, it is tissue-specific, being predominantly expressed in the brain (Schmauss et al., 1992). Research on *CWC25* is scarce, but what is

available suggests it acts as part of the spliceosome, specifically in the first catalytic reaction (Schneider et al., 2015; Tseng et al., 2017; Chiu et al., 2020).

hnRNPs combine with heterogeneous nuclear RNAs (hnRNAs) to form heterogeneous nuclear ribonucleoprotein particles (Dreyfuss, Swanson, & Piñol-Roma, 1988). hnRNPs have a variety of functions relating to nucleic acid metabolism, such as transcriptional regulation and mRNA stabilization (Geuens, Bouhy, & Timmerman, 2016). More importantly, some mediate the splicing process. Both *HNRNPK* and *PTBP1* have been discussed at length in the previous analysis for their roles in splicing, with the latter particularly noted for contributing to the alternative splicing of tau protein. Like other hnRNPs involved in splicing, *HNRNPL* binds to intronic or exonic sites in pre-mRNA, either activating or repressing exon inclusion (Rothrock, House, & Lynch, 2005; Loh et al., 2015). Interestingly, it seems to regulate the alternative splicing of potassium channels (Liu et al., 2012). Unlike the previously mentioned hnRNPs, there is no evidence signifying it has any interaction with tau.

The *Drosophila* proteins seem to share the functions of their human orthologs. *snRNP-U1-C* (also known as *U1C* or *CG5454*) is required for mRNA splicing, including in alternative splicing (Park et al., 2004; Katzenberger et al., 2009). *SmB*'s function is mainly inferred (Mount & Sal, 2000), but it is present in evolutionarily conserved spliceosome complexes (Herold et al., 2009). There is virtually no information on *Drosophila Cwc25*, so its functions have also been inferred from information available for its human counterpart. *HnRNP-K* binds to active transcription sites (Hovemann et al., 2000) and both it and *sm* appear to regulate alternative splicing (Brooks et al., 2015). Finally, *heph* binds to 3′ untranslated regions and mediates the formation RNP complexes (Besse, de Quinto, Marchand, & Trucc, 2009).

Is there a link between splicing and AD tau pathology? Regarding the hnRNPs, *PBTP1* was previously highlighted as being involved in tau-based pathology, while *HNRNPK* may have a potential connection via cytoskeletal mechanics. Furthermore, while *HNRNPL* itself does not appear to affect tau, it and other hnRNPs interact with an intermediary protein called TAR DNA-binding protein 43 (*TDP-43*). *TDP-43* inclusions have been found in several neurodegenerative disorders, including FLD and AD (Arai et al., 2009; Arai et al., 2010), and it appears to colocalise with pathogenic tau (Higashi et al., 2007), influencing the latter's aggregation (Davis et al., 2017). According to Appocher et al. (2017), both human and *Drosophila* hnRNPs affect the function of *TDP-43* if their expression levels are altered; furthermore, hnRNPs are overexpressed in FTLD-TDP-positive brains in response to loss-of-function TDP-43 (Mohagheghi et al., 2016). Whether this interaction influences pathogenic tau is uncertain.

The U1 snRNP and its components are involved in AD pathology. Bai et al. (2013) and Hales et al. (2014) observed that the protein and RNA components of U1 snRNP respectively accumulate in AD to form pathological aggregates. Additionally, knockdown of *U1C* increased levels of *APP* and *GSK3B*, though no pathogenic effects were observed (Zhu et al., 2020). This interaction appears to be bidirectional. In *Drosophila*, abnormal expression of *U1C* and *SmB* enhanced tau neurotoxicity; however, soluble tau led to a loss of snRNP proteins, in turn disrupting spliceosome function (Hseih et al., 2019).

RAB Groups

RAB proteins are small monomeric GTPases/GTP-binding proteins that act as key regulators of intracellular transport processes, including endocytosis and exocytosis (Jordens, Marsman, Kuijl, & Neefjes, 2005; Bhuina & Roy, 2014). This is particularly informative when considered alongside CM1, where proteins involved in vesicle-related processes are also

conserved. Furthermore, there are interactions between proteins of both sets of modules, an aspect covered below.

RAB proteins are not uniform in functions. *RAB2A* is required to transport proteins from the ER to the Golgi complex (Tisdale & Balch, 1996; Tisdale, 1999), as well as autolysosome formation and degradation (Lőrincz et al., 2017; Ding et al., 2019). *RAB4A* is localised to early endosomes (van der Sluij et al., 1991), and appears to be heavily involved in endosomal sorting and recycling (van der Sluijs, 1992; McCaffrey et al., 2001; Mohrmann et al., 2002). *RAB5A* is also localised to early endosomes, but in contrast to *RAB4A* it primarily mediates membrane fusion (Gorvel et al., 1991; Stenmark et al., 1994; Rybin et al., 1996). *RAB7A* mainly regulates endo-lysosomal transport (Vitelli et al., 1997; Lebrand et al., 2002; Vanlandingham & Ceresa, 2009), but it is also involved in lysosome (Bucci et al., 2000) and autophagic vacuole biogenesis (Jäger et al., 2004; Yamaguchi et al., 2009). Finally, *RAB27A* is involved in membrane trafficking (Wilson et al., 2000; Fukada, 2005) and appears to control exosome secretion (Ostrowski et al., 2009) across multiple systems, including the immune system (Stinchcombe et al., 2001; Haddad et al., 2001).

GDI1 (Rab GDP dissociation inhibitor alpha) and *VPS41* (vacuolar protein sorting-associated protein 41 homolog) and their *Drosophila* orthologs do not belong to the RAB protein family. *GDI1* acts as a regulator of RAB proteins, mediating their GDP/GTP exchange reaction and their dissociation from the membrane (Sedlacek et al., 1994; Pfeffer, Dirac-Svejstrup, & Soldati, 1995). The product of *VPS41* comprises a subunit of a protein complex known as the "Homotypic fusion and protein sorting" (HOPS) complex (Rieder & Emr, 1997; Bröcker et al., 2012). The HOPS complex primarily regulates membrane tethering in late endosomes (Balderhaar & Ungermann, 2013; Solinger & Spang, 2013), a step required before membrane fusion. Interestingly, the HOPS complex catalyses membrane fusion by assembling, binding and chaperoning the SNARE complex (Hickey & Wickner, 2010; Krämer & Ungermann,

2011; Zick & Wickner, 2013; Orr et al., 2017). Within the context of this study, this not only shows a common link between two modules, but also suggests a process ripe for further study in an AD context.

Most of the *Drosophila* proteins share the same functions as their human orthologs; however, there is more emphasis placed on their roles in the nervous system. Like *RAB2A*, *Rab2* is involved in ER-Golgi complex transport (Ke et al., 2018) and promotes autolysosome degradation (Lőrincz et al., 2017), but also drives endosome-lysosome fusion (Lund et al., 2016). Additionally, it regulates presynaptic precursor vesicle biogenesis (Götz et al., 2021), NMJ organisation (Mallik et al., 2017), and axonal transport of dense core vesicles and endolysosomal organelles (Lund et al., 2021). *Rab4*'s roles in endocytosis are inferred from its structural similarity to the paralog *Rab11*, but some experimental evidence supports its recycling function in *Drosophila* (Walsh et al., 2021); in addition, *Rab4* may also regulate synapse organization (Dey et al., 2017; White et al., 2020). *Rab5* appears to function similarly to *RAB5A*, localising to early endosomes (Lőrincz et al., 2016) and involved in membrane fusion (Morrison et al., 2008), especially in the nervous system where it fuses synaptic vesicles to the endosome (Wucherpfennig et al., 2003).

Rab7 retains its role as a regulator of endo-lysosomal transport (Entchev, Schwabedissen, & González-Gaitán, 2000; Wilkin et al., 2008), and in addition it appears to influence NMJ postsynaptic density and glutamatergic receptor levels (Patel et al., 2020; Basargekar et al., 2020). The role of *Rab27* is mostly inferred, but Corrigan et al. (2014) show it retains its function in exosome secretion, while Lien et al. (2020) found it localised to the α/β posterior neurons of the fly mushroom bodies. *Gdi* is virtually identical to *GDI1*, in that it dissociates RAB proteins from the membrane (Garrett et al., 1993; Ricard et al., 2001), though it does not appear to have any neuronal functions. Lastly, like its human counterpart *VPS45*, *lt*

encodes for a subunit that constitutes part of the *Drosophila* HOPS complex, which appears to work largely the same way (Takáts et al., 2014; Lőrincz et al., 2016).

Ribosomal Protein Complexes

Most proteins in these modules are constituents of the eukaryotic ribosome. More specifically, the *RPL* proteins are structural components of the large ribosomal subunit (60S). The sole *RPS* protein is part of the small ribosomal subunit (40S) (Anger et al., 2013).

In terms of function, the 40S subunit reads and decodes mRNA, while the 60S subunit catalyses the formation of peptide bonds (Lafontaine & Tollervey, 2001). However, these functions are due to the rRNA core rather than the proteins (Cech, 2000; Steitz & Moore, 2003), and so aside from RNA binding the exact role of ribosomal proteins in ribosome-led protein synthesis remains undetermined.Nevertheless, recent evidence has suggested that these proteins have extraribosomal functions. *RPL23A* has been linked to various biological processes outside of the ribosome, including activation of the p53 pathway (Dai et al., 2004; Jin et al., 2004) and inhibition of cell cycle arrest (Wanzel et al., 2008).

The non-ribosomal proteins, both *ETF1* and *GSPT1* (alternatively known as *ERF1* and *ERF3*, respectively) direct the termination of protein translation (Frolova et al., 1994; Zhouravleva et al., 1995; Salas-Marco & Bedwell, 2004). Their *Drosophila* orthologs, *eRF1* and *eRF3*, also possess this function (Chao, Dierick, Addy, & Bejsovec, 2003). *PPP2R1A* encodes for a regulatory subunit of protein phosphatase 2A (*PP2A*), involved in numerous biological pathways (Zolnierowicz, 2000). *PP2A* appears to associate with *ETF1*, which targets it towards the ribosomal substrates (Lechward et al., 1999); evidence suggests it regulates phosphorylation of nuclear ribosomal proteins (Kim et al., 2009). As an individual component, *PPP2R1A* appears important for chromosome segregation (Tang et al., 2006).

The *Drosophila* ortholog of *PPP2R1A*, *Pp2A-29B,* is near-identical in function as a PP2A structural protein (Ribeiro et al., 2010) and chromosome segregation (Chen et al., 2007).

General Discussion

AD is undoubtedly a complex disease: its pathology is well-characterised, but its aetiology is still not largely understood. As this study illustrates, the progression of the disease involves numerous biological processes that become dysfunctional during the initiation and propagation of AD pathology. This is especially true for tau-related pathology, and as my study shows no individual process stands out as the primary "trigger" in either humans or *Drosophila*. Comparing two species with an evolutionary divergence of approximately 797 million years (according to TimeTree; Hedges, Dudley, & Kumar, 2006) is, however, informative with the number of biological processes and individual proteins involved in AD pathology conserved across both humans and *Drosophila,* revealing that the progression of tau-induced neurodegeneration involves dysfunction in largely the same basic biological processes and suggesting that the progression of the disease involves the disruption of fundamental cellular processes. According to my results, vesicle-related and protein kinase processes are the most commonly disturbed in both human and *Drosophila* AD.

Furthermore, it also suggests that animal models will remain in use as powerful tools in understanding AD and that increased use of omics technologies will continue to elucidate the mechanics of the disease. However, my study does also illustrate the importance of understanding the limitations and differences of the different model organisms. This is exemplified by the fact that the immune system plays a role in mammalian models of AD, but contributes little to nothing in *Drosophila* despite the fact that immune system processes play a key part in the progression of the disease in mammals.

In undertaking this study, I sought to analyse the published literature to further understand the biological processes involved in tauopathies. To do this, I drew on a large volume of published data that whilst of high quality did occasionally cause some complications in my study.

To ensure a high-quality analysis my sourcing of data was consistent and analysed papers were selected on stringent criteria, as detailed in the methods section. The genomic and transcriptomic data from these studies were isolated used standardised experimental protocols, albeit with some minor variations. Whilst the studies used different species, these could easily be divided into being either "mammalian" or "arthropod" and finding orthologous genes in humans and *Drosophila* was significantly easier than if a wider range of organisms had been used (e.g. *Danio rerio*). Furthermore, the studies were all consistent in that most genes/proteins identified had annotated functions in biological databases such as UniProt and FlyBase, making the analyses, specifically the GO analysis, easier to complete.

Whilst the core data in these studies was consistent, reporting of significantly changed genes/proteins between studies was occasionally inconsistent. In particular the description of fold differences values (i.e. whether a protein/gene was positively or negatively expressed) was variable. Some studies reported values in logarithmic format, whilst others reported them in raw or undiscernible formats. A few did not explicitly describe gene/protein expression levels, only emphasising significant genes/proteins descriptively within the text. As a result, a heuristic approach was required to highlight and extract genes/proteins of interest, as described in the methods. This resulted in a mixture of quantitively (i.e. significance values or fold differences) and qualitatively (i.e. descriptively significant) selected data; ideally, this data should have been identified and isolated by purely quantitative values but this would

have meant discarding key studies. There was also a lack of gene/protein name consistency, though this is a systemic problem given that gene/protein nomenclature is dynamic.

Another issue was that the annotation of *Drosophila* genes is biased towards developmental processes, reflecting the use of *Drosophila* as a model for developmental biology, with most gene functions often observed within this context. Nevertheless it is likely they share similar functions to their human orthologues, but have not have been studied outside of developmental processes. Despite these minor issues, this study has revealed powerful and informative results that can provide further insight into the disease processes behind AD and other tauopathies. The most striking outcome from my analyses is the number of biological processes and individual proteins involved in AD pathology conserved across both humans and *Drosophila*.

Whilst the identification of conserved genes and proteins underlying the disease process is a powerful step forwards, one of the key messages to arise from my study is the realisation that diseases are not static processes, but dynamic ones that can change rapidly or slowly over a given amount of time and differ from species to species. This study has shown that many genes/proteins implicated in AD pathology operate within a network of protein interactions with impacts on an even wider array of networks. In my opinion, the biggest conclusion to draw from my study is that AD is a disease of multiple systems, all which function abnormally and in tandem to seemingly converge on tau protein.

In this context the conserved module analyses especially strengthen this point, revealing conserved, highly connected subnetworks of proteins with specific functions that can be further studied within the context of AD. This supports the notion that the foremost approach to study AD might be to not to focus on single proteins, or even several individual proteins acting separately, but rather focus on methodologies to analyse the multiple interacting

pathways that converge and diverge as the disease progresses. For example, multiple kinases regulate the RNA splicing of tau (Hernández et al., 2004; Shi et al., 2011), which in turn is propagated by vesicle-traffic related processes (Wu et al., 2013; Zhou et al., 2017).

My networks studies clearly point to processes that have been highlighted as particularly interesting. Processes relating to vesicles, for example, may prove fruitful avenues of research in future AD studies, considering their near ubiquity across all studies, albeit to varying degrees. There is also evidence that RNA splicing, both the core process and its regulation, is aberrantly affected during tau-specific pathology. Current knowledge regarding the *normal* functions of these subnetworks is lacking, especially in *Drosophila* where many proteins and their functions are assumed via phylogenetic similarity. Establishing how these proteins normally function would aid in understanding their role in AD. Further suggestions for further research conducted in this area should aim to elucidate the protein-protein interactions within and between these subnetworks, both as individual proteins and as whole systems, and it should be done in both *Drosophila* and humans to establish an empirical basis for the potential evolutionary link found in these analyses.

Of course, this does not mean that studying individual proteins has no value, but they should ideally be studied within the context of a larger process. *EGFR/Egfr*, *SRC/Src64B*, *DLG4/dlg1*, *SOD2/Sod2*, *ITGB1/mys*, *EPHA1/Eph*, *CTSD/cathD*, and *BIN1/Amph* stand out as candidates for further tau-based pathology studies, owing to their significance in the centrality analyses of both species. *EGFR/Egfr*, *SRC/Src64B*, and *EPHA1/Eph* may be especially interesting for further analysis, considering the enrichment of GO functions relating to protein kinases in both species and the already-established role of protein kinases in tau pathogenesis. In future research, using the module conservation approach could also determine whether protein kinases form interspecies functional complexes.

Regarding cellular location, many proteins in this study act in neuronal substructures, including the synapses and dendritic processes. This is perhaps the least surprising result, given that AD is exclusively neurological, but it does further confirm the presence of synaptic dysfunction in the disease. More intriguingly, proteins that act within or adjacent to ribosomes are conserved across humans and *Drosophila*, exemplified by the CM4 module. It may prove worthwhile to elucidate the role of ribosomes in tau pathology in the future, as well as the potential role of other organelles in AD.

To conclude, I believe this study has successfully identified and emphasised potentially conserved mechanisms in AD that may serve as bases for future studies, especially those employing *Drosophia* as AD modes. The interaction of protein kinases with vesicle-related processes and RNA splicing may prove to be fruitful avenues for exploration.

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Appendix A

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Appendix B

List of human genes and *Drosophila* orthologues identified as significant

ABCA1/CG34120	*ATP5F1/ATPsynB*	*CCT8/CCT8*
ABCA7/CG8908	*ATP5H/ATPsynD*	*CD22/ed*
ABCC2/MRP	*ATP6V1F/Vha14-1*	*CD2AP/cindr*
ABCG1/Atet	*B3GALT2/GalT1*	*CD33/hbs*
ACBD5/CG8814	*BACE1/CG31926*	*CD52*
ACE/Ance	*BDNF*	*CD68/Lamp1*
ACOT1/Bem46	*BECN1/Atg6*	*CDC20/fzy*
ACTB/Act42A	*BHLHE22/Oli*	*CDH23/ds*
ADAM10/kuz	*BIN1/Amph*	*CDH5/CadN2*
ADAM33/Meltrin	*BMS1P7*	*CDK17/Eip63E*
AGTPBP1/CG31019	*C4B/Mcr*	*CELF1/bru1*
ALDH18A1/CG7470	*CACNA1B/cac*	*CELF2/bru1*
AMPH/Amph	*CALM3/Cam*	*CENPE/cmet*
ANK1/Ank	*CAMK1D/CaMKI*	*CHD2/Chd1*
ANKEF1/CG30271	*CAMK2D/CaMKII*	*CHD3/Mi-2*
ANKRD49/l(2)35Be	*CAMK2N1/*	*CHRNB2/nAChRbeta1*
AOX1/ry	*CAMK4/CG17528*	*CLEC7A/CG14866*
APOC1	*CAMKK2/CG17698*	*CLU*
APOE	*CAPN1/CalpA*	*COL17A1*
APP/Appl	*CAPN2/CalpB*	*CPLX3/cpx*
AQP1/Prip	*CASS4/p130CAS*	*CR1/hasp*
ARC/Arc2	*CAST*	*CRH/fw*
ARV1/Arv1	*CCNE2/CycE*	*CSDA*

CST3/Cys

CST7/CG31313

CTSD/cathD

CWC25/Cwc25

CX3CR1/AstA-R1

CYTC1

DAPK2/CG31345

DCHS2/ds

DEX

DIP2A/DIP2

DLG4/dlg1

DNAH17/Dhc93AB

DNAJB5/CG5001

DOCK2/mbc

DPF3/d4

DRAP1/NC2alpha

DTNA/Dyb

DUSP1/puc

DUSP4/puc

DYNC1LI1/Dlic

EEF1A1/eEF1alpha1

EGFR/Egfr

EHD4/Past1

EIF4BP9

EPHA1/Eph

EPHA4/Eph

ERCC4/mei-9

ESR1/ERR

ETF1/eRF1

FABP7/fabp

FBP1/fbp

FCER1G/

FERMT2/Fit1

FOSB/kay

FRK/Src42A

FYN/Src64B

GABBR1/GABA-B-R1

GAPDHS/Gapdh1

GCC2/GCC185

GDI1/Gdi

GFAP/LamC

GLIS3/lmd

GMNC/geminin

GOLGA8A/GM130

GOLGA8B/GM130

GPHN/cin

GPM6B/M6

GPX1/CG15116

GRB14/pico

GRIA1/GluRIA

GRIA2/GluRIA

GRIK1/Ekar

GRIK3/KaiR1D

GRIPAP1

GRM4/mGluR

GRM6/mGluR

GSK3B/sgg

GSPT1/eRF3

GSTA4/GstS1

H2AFZ/His2Av

HDLBP/Dp1

HIP1/Hip1

HLA-DRB1/tefu

HLA-DRB5/

HMHA1

HNRNPK/HnRNP-K

HNRNPL/sm

HNRPLL/sm

ICA1/ICA69

IDE/Ide

IGF2R/Lerp

IL1A

IL1B

IL1RAP/otk

IL34

INPP5D/CG6805	*LPAR3*	*MYO5C/didum*
IQCK	*LRP6/arr*	*NCSTN/Nct*
ITGA3/mew	*LY6H*	*NEUROD1*
ITGA8/if	*LYZ1*	*NEUROD6*
ITGA9/ItgaPS5	*LYZ2*	*NFH*
ITGAM/if	*MAB21L2/CG4766*	*NFIC*
ITGAX/if	*MACROH2A1*	*NPAS4/dysf*
ITGB1/mys	*MADD/Rab3-GEF*	*NPC2/Npc2a*
ITPKB/IP3K2	*MAP1B/futsch*	*NR4A2/Hr38*
KCNA2/Sh	*MAP3K1/*	*NR5A1/ftz-f1*
KCNAB1/Hk	*MARK1/par-1*	*NRF1/ewg*
KCND2/Shal	*MAST4/dop*	*NRN1*
KIAA1522/	*MDM2/Non2*	*NTN1/NetB*
KIF11/Klp61F	*MEF2C/Mef2*	*NTRK2*
KIFAP3/Kap3	*MFAP1/Mfap1*	*NTRK3*
KLK3/CG16749	*MMP17/Mmp2*	*NUCKS*
KMT2C/trr	*MOSPD3/fan*	*NUP98/Nup98-96*
KPNB1/Fs(2)Ket	*MPP4/metro*	*NVL/smid*
KSR1/ksr	*MS4A4A*	*OPTN/key*
L1CAM/Nrg	*MS4A4E*	*OR13G1*
LAMP1/Lamp1	*MS4A6A*	*OSMR*
LEFTY1/daw	*MS4A7*	*OSTN*
LMAN1/ergic53	*MTHFR/CG7560*	*PARD6G/par-6*
LOC400891/CG7886	*MTND4LP1*	*PCDH8*
LOC51231	*MUM1*	*PFKL/Pfk*

PFKM/Pfk	*PTTG1*	*RWDD2A/CG30338*
PFKP/Pfk	*PVALB*	*SAP18/Bin1*
PHYHD1/Phyhd1	*PVR*	*SCN11A/para*
PICALM/lap	*PVRL2*	*SCN2A2*
PLCG2/sl	*RAB24*	*SCRIB/scrib*
PLOD3/Plod	*RAB27/Rab27*	*SDE2/CG5986*
PPP1R3B/Gbs-70E	*RAB2A/Rab2*	*SDHA/SdhA*
PPP2R1A/Pp2A-29B	*RAB4/Rab4*	*SDHB/SdhB*
PPP2R3C	*RAB5/Rab5*	*SEC22B/Sec22*
PPP3CA/CanA-14F	*RAB7/Rab7*	*SEMA4C/Sema2a*
PRDX3/Prx3	*RBM45/CG1316*	*SERPINA1/Spn28Dc*
PRKCB/Pkc53E	*RBM6/CG4896*	*SERPINA3/Spn28Dc*
PRKCG/Pkc53E	*RELN*	*SERPINA3N/Spn28Dc*
PRNP	*RGS4*	*SERPINA5/Spn28Dc*
PRSS12	*RHBDF2/rho-5*	*SERPINF1/Spn42De*
PRSS27	*RIMS2/Rim*	*SERPINF2/Spn75F*
PSEN1/Psn	*RIN3/spri*	*SFN/14-3-3zeta*
PSMB2/ProsÎ²5	*RPH3A/Rph*	*SLC1A1/Eaat1*
PSMB8/ProsÎ²5	*RPL13/RpL13*	*SLC24A4/zyd*
PSMC3IP	*RPL15/Rpl15*	*SLC35D2/frc*
PTBP1/heph	*RPL23A/RpL23A*	*SLC38A2/mah*
PTGS2	*RPLP0/RpLP0*	*SLC6A1/Gat*
PTK2B/Fak	*RPN1/CG33303*	*SLC7A2/slif*
PTMA	*RPS21/RpS21*	*SLCO4A1/Oatp26F*
PTPRD/Lar	*RUNX1/lz*	*SNAP23/Snap24*

SNCA	*SYTL5/btsz*	*TREM2*
SNRPC/snRNP-U1-C	*TAC1/Tk*	*TREML2*
SNRPN/SmB	*TECR/Sc2*	*TRIL/haf*
SOD2/Sod1	*TESPA1*	*TRMT2A/CG3808*
SORL1	*TF/Tsf2*	*TYROBP/Galphaf*
SPPL2A	*TFAM/TFAM*	*UTRN/Dys*
SRC/Src64B	*TFAP4/crp*	*VAMP1/Syb*
SST	*TIMELESS/timeout*	*VAPB/Vap33*
STIP1/Stip1	*TJP1/pyd*	*VMP1/Tango5*
STX4A/Syx4	*TJP2/pyd*	*VPS41/lt*
STX7/Syx7	*TNF*	*WNK1/Wnk*
SYN3/Syn	*TOMM40/tom40*	*XYLT1/oxt*
SYNJ1/Synj	*TP53AIP1*	*ZCWPW1*
SYNPO/CG1674	*TPBG/CG6959*	*ZFP36/Tis11*
SYP	*TRAK1/milt*	

Appendix C

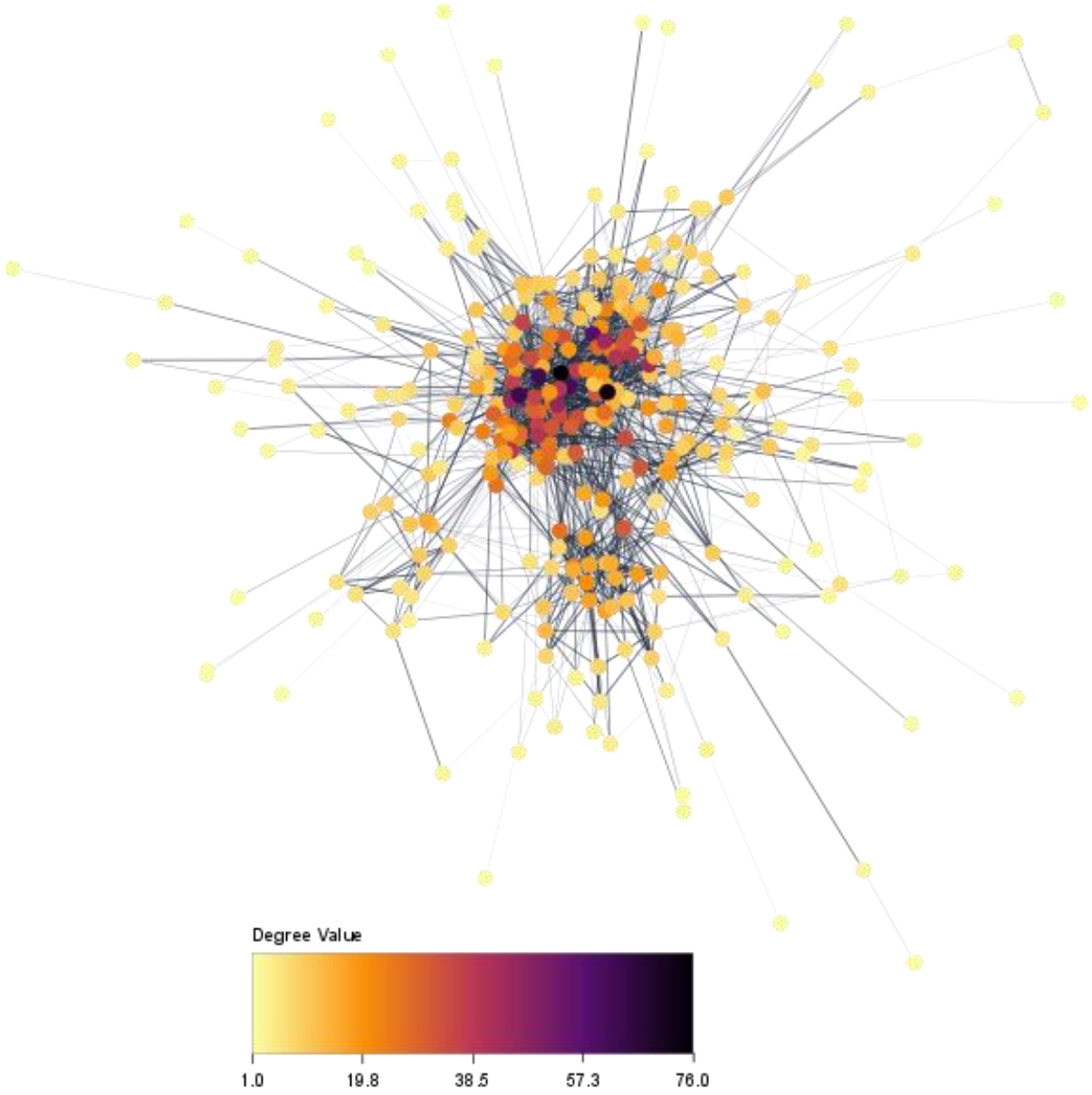
Table of references used to approximate GO terms for specific gene/protein functions

Gene	Function	Evidence
Amph	Cytoskeleton organization	Dräger, N., Nachman, E., Winterhoff, M., Brühmann, S., Shah, P., & Katsinelos, T. et al. (2017). Bin1 directly remodels actin dynamics through its BAR domain. EMBO Reports, 18(11), 2051-2066. doi: 10.15252/embr.201744137
Amph	Membrane organization	Zelhof, A. C., Bao, H., Hardy, R. W., Razzaq, A., Zhang, B., & Doe, C. Q. (2001). *Drosophila* Amphiphysin is implicated in protein localization and membrane morphogenesis but not in synaptic vesicle endocytosis. Development (Cambridge, England), 128(24), 5005–5015. Zhang, B., & Zelhof, A. (2002). Amphiphysins: Raising the BAR for Synaptic Vesicle Recycling and Membrane Dynamics. Traffic, 3(7), 452-460. doi: 10.1034/j.1600-0854.2002.30702.x
Eph	Axon guidance	Boyle, M. (2006). *Drosophila* Eph receptor guides specific axon branches of mushroom body neurons. Development, 133(9), 1845-1854. doi: 10.1242/dev.02353
Eph	Regulation of cell population proliferation	Franco, M., & Carmena, A. (2019). Eph signaling controls mitotic spindle orientation and cell proliferation in neuroepithelial cells. Journal Of Cell Biology, 218(4), 1200-1217. doi: 10.1083/jcb.201807157
Eph	Pattern specification process	Porazinski, S., de Navascués, J., Yako, Y., Hill, W., Jones, M., & Maddison, R. et al. (2016). EphA2 Drives the Segregation of Ras-Transformed Epithelial Cells from Normal Neighbors. Current Biology, 26(23), 3220-3229. doi: 10.1016/j.cub.2016.09.037
Fak	Cell adhesion	Ribeiro, S., D'Ambrosio, M., & Vale, R. (2014). Induction of focal adhesions and motility in *Drosophila* S2 cells. Molecular Biology Of The Cell, 25(24), 3861-3869. doi: 10.1091/mbc.e14-04-0863
Fak	Cell migration	Fox, G., Rebay, I., & Hynes, R. (1999). Expression of DFak56, a *Drosophila* homolog of vertebrate focal adhesion kinase, supports a role in cell migration in vivo. Proceedings Of The National Academy Of Sciences, 96(26), 14978-14983. doi: 10.1073/pnas.96.26.14978
Fak	Negative regulation of kinase activity	Macagno, J., Diaz Vera, J., Yu, Y., MacPherson, I., Sandilands, E., & Palmer, R. et al. (2014). FAK Acts as a Suppressor of RTK-MAP Kinase Signalling in *Drosophila* melanogaster Epithelia and Human Cancer Cells. Plos Genetics, 10(3), e1004262. doi: 10.1371/journal.pgen.1004262

Arc2	Long-term memory	Awata, H., Takakura, M., Kimura, Y., Iwata, I., Masuda, T., & Hirano, Y. (2019). The neural circuit linking mushroom body parallel circuits induces memory consolidation in *Drosophila*. Proceedings Of The National Academy Of Sciences, 116(32), 16080-16085. doi: 10.1073/pnas.1901292116
Arc2	Regulation of synaptic plasticity	Ashley, J., Cordy, B., Lucia, D., Fradkin, L., Budnik, V., & Thomson, T. (2018). Retrovirus-like Gag Protein Arc1 Binds RNA and Traffics across Synaptic Boutons. Cell, 172(1-2), 262-274.e11. doi: 10.1016/j.cell.2017.12.022
CaMKI	Protein phosphorylation	Kahn, E., & Matsumoto, H. (2002). Calcium/Calmodulin-Dependent Kinase II Phosphorylates *Drosophila* Visual Arrestin. Journal Of Neurochemistry, 68(1), 169-175. doi: 10.1046/j.1471-4159.1997.68010169.x
Cindr	Actin cytoskeleton organization	Johnson, R., Seppa, M., & Cagan, R. (2008). The *Drosophila* CD2AP/CIN85 orthologue Cindr regulates junctions and cytoskeleton dynamics during tissue patterning. Journal Of Cell Biology, 180(6), 1191-1204. doi: 10.1083/jcb.200706108
Lerp	Lysosome organization	Hasanagic, M., van Meel, E., Luan, S., Aurora, R., Kornfeld, S., & Eissenberg, J. (2015). The lysosomal enzyme receptor protein (LERP) is not essential, but is implicated in lysosomal function in *Drosophila* melanogaster. Biology Open, 4(10), 1316-1325. doi: 10.1242/bio.013334
Pfk	Glycolysis	Currie, P., & Sullivan, D. (1994). Structure and expression of the gene encoding phosphofructokinase (PFK) in *Drosophila* melanogaster. Journal Of Biological Chemistry, 269(40), 24679-24687. doi: 10.1016/s0021-9258(17)31444-8

Appendix D

Image of full-scale human AD network



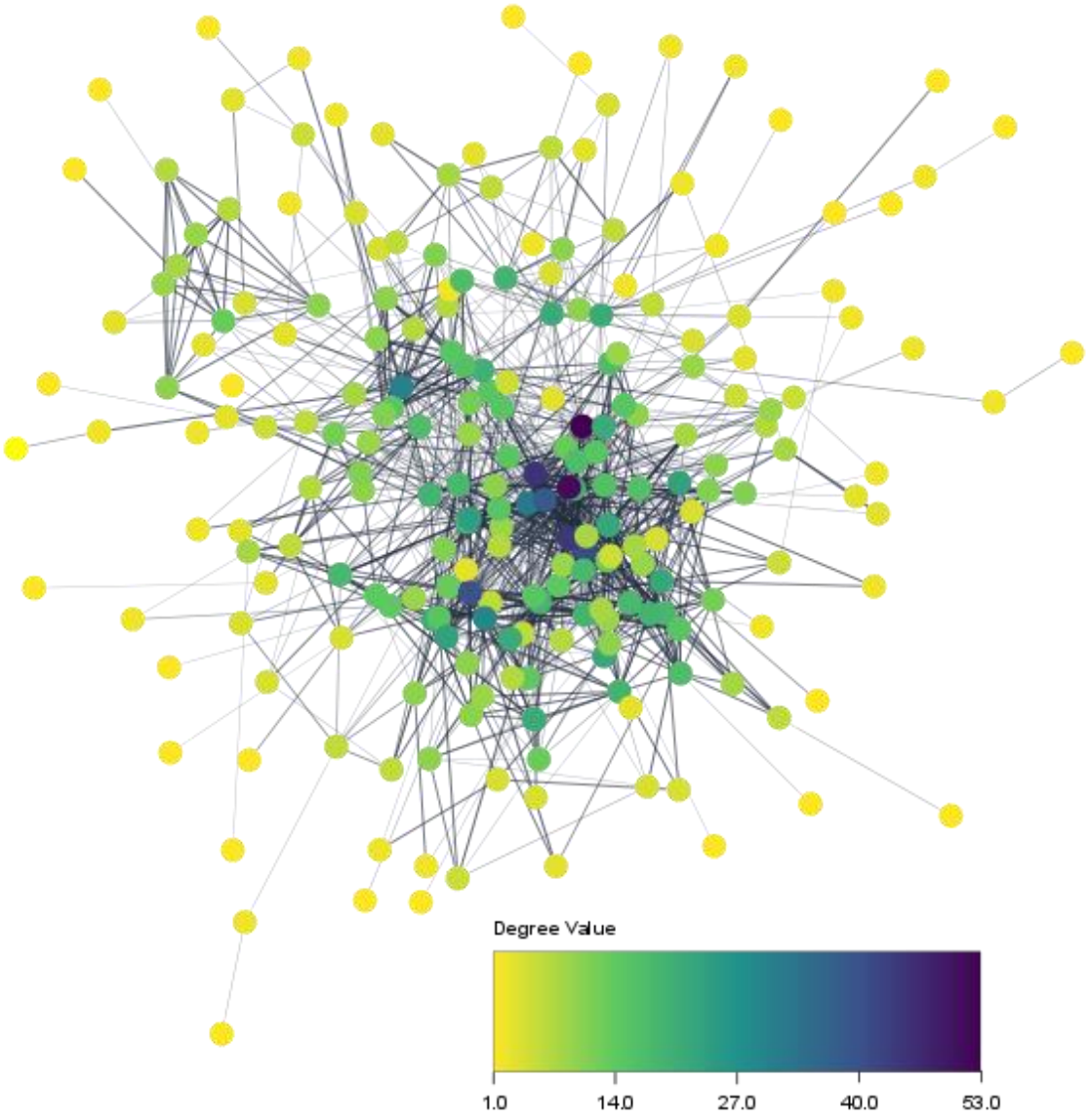
Appendix E

Code used to generate random networks and to compare with human AD centrality values

```
#loading the relevant libraries/packages
library(igraph)
library(ggplot2)
library(dplyr)

#set seed for reproducibility
set.seed(1110)

#generation of multiple random graphs for human comparison
er_graph_multiple <- sample_gnm(307,2007, directed = FALSE, loops =
FALSE)*1000

#calculate centralities for random networks
er_k_value <- degree(er_graph_multiple)
er_bc_value <- betweenness(er_graph_multiple)
er_trans_value <- transitivity(er_graph_multiple,type = "local")

#sample number of values from each centrality
sample_er_k <- sample(er_k_value,307,replace = FALSE)
sample_er_bc <- sample(er_bc_value,307,replace = FALSE)
sample_er_trans <- sample(er_trans_value,307,replace = FALSE)

#import centrality data for the AD human network
ad_human_graph <- read.csv("ad_human_centralities.csv",header = TRUE)

#create separate values for AD human centrality measures
ad_human_k_value <- ad_human_graph$degree
ad_human_bc_value <- ad_human_graph$betweenness
ad_human_trans_value <- ad_human_graph$clust_coeff

#different Wilcoxon rank sum tests
wilcox.test(ad_human_k_value,sample_er_k,paired = TRUE)

/Wilcoxon signed rank test with continuity correction

data:  ad_human_k_value and sample_er_k
V = 18776, p-value = 0.01149
alternative hypothesis: true location shift is not equal to 0

wilcox.test(ad_human_bc_value,sample_er_bc,paired = TRUE)

/Wilcoxon signed rank test with continuity correction

data:  ad_human_bc_value and sample_er_bc
V = 28755, p-value = 0.001015
alternative hypothesis: true location shift is not equal to 0
```

Appendix F

Image of full-scale *Drosophila* AD network



Appendix G

Code used to generate random networks and compare with *Drosophila* AD centrality values

```
#loading the relevant libraries/packages
library(igraph)
library(ggplot2)
library(dplyr)


#generation of multiple random graphs for Drosophila comparison
er_graph_multiple <- sample_gnm(307,2007, directed = FALSE, loops =
FALSE)*1000

#calculate centralities for random networks
er_graph_multiple <- sample_gnm(224,1080, directed = FALSE, loops =
FALSE)*1000
er_k_value <- degree(er_graph_multiple)
er_bc_value <- betweenness(er_graph_multiple)

#sample number of values from each centrality
sample_er_k <- sample(er_k_value,224,replace = FALSE)
sample_er_bc <- sample(er_bc_value,224,replace = FALSE)

#import centrality data for the AD Drosophila network
ad_dros_graph <- read.csv("ad_Drosophila_centralities.csv",header = TRUE)

#create separate values for AD Drosophila centrality measures
ad_dros_graph <- read.csv("ad_Drosophila_centralities.csv",header = TRUE)
ad_dros_k_value <- ad_dros_graph$Degree.unDir
ad_dros_bc_value <- ad_dros_graph$Betweenness.unDir

#different Wilcoxon rank sum tests
wilcox.test(ad_dros_bc_value,sample_er_bc,paired = TRUE)

/Wilcoxon signed rank test with continuity correction

data:  ad_dros_bc_value and sample_er_bc
V = 15032, p-value = 0.01228
alternative hypothesis: true location shift is not equal to 0

wilcox.test(ad_dros_k_value,sample_er_k,paired = TRUE)

/Wilcoxon signed rank test with continuity correction

data:  ad_dros_k_value and sample_er_k
V = 10280, p-value = 0.04713
alternative hypothesis: true location shift is not equal to 0
```